APTITUDE IN AMERICAN ALLIGATORS:

ECOLOGICAL FACTORS AFFECTING

COGNITION AND BEHAVIOR

by

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ABSTRACT

American alligators (*Alligator mississippiensis*) possess flexible cognitive abilities. Given these cognitive abilities, we hypothesized the capacity for flexible learning is significantly affected by ecological factors such as environmental temperature and contaminants. Additionally, we reasoned that the same ecological modifying inherent cognitive capacities also shape other traits in adaptive ways. In order to explore these relationships we utilized three separate studies divided between four chapters. In the first three chapters our research employed serial-reversal experiments to quantify the total number of errors in a series of discrimination problems to investigate the effect of temperature and environmental contaminates on inherit cognitive abilities. Chapter 4 is devoted to an investigation of the effect of these same environmental contaminates on thermoregulatory behavior and metabolism. Specifically, experiments presented in chapter one investigated the visual learning abilities of juvenile American alligators in order to determine the degree of complex learning. Chapter 2 describes the effect of temperature on the performance of juvenile American alligators in a spatial discrimination task by tasking animals to complete a series of ten reversal at two environmentally relevant temperatures. The successful development of both protocols led us to ask the question of whether or not we could apply a similar behavioral assessment of learning and memory abilities in

animals exposed to environmental contaminants. Recognizing that organochlorines still contaminate the waters inhabited by American alligators, Chapter 3 explores the affect *in ovo* exposure to DDE has on learning and behavior in hatchling American alligators. Specifically, we investigated if DDE affects cognition of American alligators by comparing the performance of individuals that were exposed as embryos to the performance of control individuals in a spatial discrimination task. Organochlorines, such as DDE, can bioaccumulate and are therefore particularly problematic for top predators. Therefore, it is important to know if standard metabolism or preferred body temperatures are perturbed by DDE exposure because these changes may affect the overall health of animals, their reproductive success, and the health and growth rates of hatchlings. Therefore, in Chapter 4 we designed a study to measure changes in the thermoregulatory system and metabolism of animals exposed to DDE.

This work is dedicated to my grandfather, Albert Araneo, for all his love and support. He is the foundation of my family and without him we would not be who we are.

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CHAPTER 1

INTRODUCTION

Several features of cognition have evolved in similar ways in both primates and several phyletically-independent species of birds (Lefebure et al., 2004). Furthermore, in birds and mammals specialized behaviors such as tool use and parental care are linked to the evolution of cognitive abilities (Lefebure and Sol, 2008). Such observations lead one to wonder if there are ecological and life-history factors that have influenced the convergent evolution of cognitive abilities in two such divergent lineages. Examples of such ecological pressures or life history patterns include food type, social groups, and climate.

Species that take advantage of a variety of feeding sources, or have to use more complicated strategies to locate or handle food, will rely on more sophisticated adaptability patterns. Furthermore, food type may force such species to rely more heavily on innovative behaviors in order to access and survive on complex food sources. For example, caching behaviors require extensive spatial memory in order to store and retrieve food (Harvey et al., 1980). Additionally, predatory behaviors require a greater ability to pursue, detect and manipulate prey (Glitterman, 1986; Huber et al., 1997). A second life history pattern that has influenced the evolution of cognitive abilities in birds and mammals is sociality. The size of a social group can influence cognitive abilities

because larger groups require greater cognitive abilities in order to keep track of group members and the social interactions and relationships between group members (Dundar, 1998). Furthermore, sociality requires group members to process and correctly respond to other group members using either visual or vocal communication signals. Such communication requires higher brain function and cognitive abilities. Finally, climate has been hypothesized to play a role in the evolution of complex behavior and cognitive abilities. Specifically, species from temperate environments have evolved to cope with an environment that can be dramatically different from one season to the next. For example, these species deal with winter temperatures that are much lower than the temperatures experienced in the summer months. Furthermore, these environments may be much harsher due to short days and food shortages that also accompany low temperatures. Innovative behavior and the ability to adapt to novel situations could improve the ability of these animals to survive and therefore increase species persistence. While these patterns are observed in birds and mammals, ectotherms live in these same environments and are sensitive to some of the same evolutionary pressures as their endothermic neighbors.

Ecological factors such as food type and social group size could influence the cognitive abilities of ectotherms in the same manner as they influence birds and mammals. For example, predators will need to be able to learn how to track and manipulate sparse and evasive prey in order to be successful, whether the predator is an ectotherm or endotherm. Similarly, social groups will still require a greater amount of neural ability to process the interactions between group

members regardless of whether the group members are ectotherms or endotherms. However, ecological factors such as environment and climate may have a very different effect on the cognitive abilities of ectotherms. Studies on ectotherms may provide new insights into the evolution of cognitive abilities because certain ecological and environmental aspects will affect ectotherms in ways that birds and mammals are insensitive. Crocodilians make a good study organism for cognitive studies because they share certain life history factors and specialized behaviors with birds and mammals (i.e., parental care, social groups and predatory behavior). However, as ectotherms they will offer unique insights into the evolution of complex behavior, adaptability to novel situations and cognitive abilities.

Crocodilians display a repertoire of complex vocal and behavioral communication cues (Modha, 1967; Garrick and Lang, 1977; Garrick et al., 1978). Both vocal and behavioral communication cues are highly developed and important for social interactions including sexual competition, territory establishment, mate selection and copulation (Garrick and Lang, 1977). Such complex interactions lend support to the idea that this species poses flexible learning abilities. Additionally, female alligators return to the same nest site at the beginning of each reproductive cycle (Elsey et al., 2008), requiring the ability to learn and remember the location of these nest sites. With a complex social structure, long-term territory establishment, parental care that includes nurturing and rearing young and adaptability to their environment, it is clear that alligators

have the capacity to learn and remember. However, the degree of this learning ability has not been suitably tested in the wild or in laboratory captive animals.

The overarching hypothesis of this thesis is that American alligators (*Alligator mississippiensis*) possess flexible cognitive abilities. Specifically, American alligators rely on visual and spatial discrimination abilities in their behavior and learning abilities, even when reared in captivity. We approached our investigation with three separate studies, described in the four chapters below. In the following three chapters, our research employed reversal problems to quantify the total number of errors in a series of discrimination problems with chapter five devoted to an investigation of thermoregulatory behavior and metabolism. Progressive improvement in the number of errors committed during a series of discrimination tasks can be taken as a measure of learning, and is observed in a variety of species (Bitterman, 1965a; 1965b).

Reversal problems are a valuable and established tool for evaluating and comparing the learning abilities of different species (Stettner et al., 1967). Futhermore, reversal problems lend insight into general problem-solving abilities "that transcends behavioral domains and different ecological demands" pg.136 (Lefebure et al., 2004). The observation that various species perform differently in these tasks lends insight into phylogenic differences in behavior and learning abilities (Stettner et al., 1967). Across several orders of birds it has been shown that the total number of errors committed in a series of visual discrimination problems declines as an individual's experience with the problem increases. Chickens (*Gallus gallus domesticus*), pigeons (*Columba livia domestica*) and

crows (*Corvus americanus)* all progressively improve in the number of errors committed during a series of visual discrimination tasks. However, in quail (*Colinus virginianus)* experience does not seem to affect the number of errors and therefore, quail do not reflect the pattern of decreasing errors seen in other bird species. We hypothesize that American alligators (*Alligator mississippiensis)* display a pattern of progressive improvement in reversal problems, similar to the pattern seen in many bird species including Corvids.

Experiments presented in Chapter 2 investigated the visual learning abilities of juvenile American alligators reared in the laboratory. Specifically, the research established optimal conditions for training and reversal using a food reward, ascertained the visual discrimination ability of the American alligator, and finally, determined the degree of complex learning using a serial reversal approach. Very little is known about the ability of American alligators to participate in these types of tasks, and the laboratory setting permits testing of the innate ability of the subject. We believe that the visual discrimination task explained in Chapter 2 closes this gap.

Chapter 3 describes the effect of temperature on the performance of juvenile American alligators in a spatial discrimination task. Based on information obtained in other species (Reid, 1957; Warren et al., 1960; Eskin and Bitterman, 1961; Northcutt and Heath, 1973), we investigated the effect of temperature on the spatial discrimination ability of American alligators. This assessment of spatial learning incorporated two different temperature treatments - one at the lower end and another at the upper end of the American alligator's preferred

activity range. The results of this study present new data and a novel approach to quantifying learning in a predatory reptile. American alligators are capable of learning to perform a lever pressing action in order to receive a food reward (Araneo and Farmer, unpubl.). Furthermore, American alligators are capable of discriminating two stimuli on the basis of visual cues (Araneo and Farmer, unpubl.). As Krekorian et al. (1968) demonstrated, desert iguanas perform better in a learning task when close to their preferred body temperature. Additionally, learning in this species appeared to be less effective at cooler temperatures (Krekorian et al., 1968). American alligators have a preferred body temperature, after feeding, of 30°C (Farmer et al., 2008). We predicted that American alligators would show differences in a spatial discrimination study based on different temperature regimes.

Temperature affects various aspects of learning in a variety of species (Roussel et al., 1982). Even with extensive training sessions, both hypothermia and hyperthermia perturb memory acquisition in the rodent (Roussel et al., 1982). Furthermore, in rats a core body temperature increase of 2 or 3°C can cause amnesia (Misanin et al., 1979). In hummingbirds, it is hypothesized that the associated drop in temperature during torpor is incompatible with memory consolidation (Roth et al., 2010). Such a trade-off, between energy conservation and memory consolidation, implies that in the hummingbird, memory consolidation may not be temperature compensated. Therefore, if juvenile American alligators resemble the pattern observed in mammals and the

hummingbird, we might expect to see an effect of temperature on the spatial discrimination ability of juvenile American alligators.

Whereas in Chapter 2 we successfully designed a learning protocol that allowed us to investigate the visual discrimination ability of juvenile American alligators, in Chapter 3 we applied this knowledge to a second learning assay that allowed us to investigate the effect of temperature on the spatial discrimination ability of this species. The effective establishment of both of these protocols led us to ask the question of whether or not we could apply a similar behavioral assessment of learning and memory abilities in animals exposed to environmental contaminants *in ovo* (Chapter 4).

The synthetic pesticide DDT [1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane] is used throughout the world, exposing humans and wildlife to this organochlorine (Kleinow et al., 1987). This pollutant and its breakdown products are linked to a variety of morphological, developmental, and physiological abnormalities (Schantz and Widholm, 2001). Even though it is known that these compounds derail normal cerebral function in birds and other species, little is known about the effects of organochlorines on the crocodilian brain (Hunt and Hunt, 1977; Luoma, 1992; Iwaniuk et al., 2006)).

Recognizing that organochlorines still contaminate the waters inhabited by American alligators, the effect of these pollutants on alligator development, survival and species preservation needs addressing. Chapter 4 explores the effect *in ovo* exposure to DDE (1,1-dichloro-2, 2-bis[p-chlorophenyl] ethylene) has on learning and behavior in hatchling American alligators. Specifically, we

investigated if organochlorines, such as DDE, affect cognition of American alligators by comparing the performance of individuals that were exposed as embryos to an organochlorine, to the performance of control individuals in a spatial discrimination task. Understanding the effects of organochlorine exposure on crocodilian cognition and behavior is important in a number of ways. Crocodilians have complex social behaviors including, territory defense, parental care and nest site fidelity. These behaviors may be critical for the health of hatchlings and persistence of the species.

An individual's fitness depends on a suite of traits that interact with the environment. How integrated phenotypes evolve that are complex and multifunctional is a central question at the frontier of evolutionary biology. We hypothesized that there would be a relationship between the capacity for flexible learning, and environmental temperature and contaminants, reasoning that the multiple ecological drivers of cognitive capacities will also shape other traits in adaptive ways. For example, if enhanced cognitive abilities increase the amount of food an individual procures, there may be selective benefits to the co-evolution of more rapid rates of growth and higher body temperature set-points. The way these phenotypes intertwine could be derailed by exposure *in ovo* to environmental contaminants. Therefore, in Chapter 5 we designed a study to measure changes in the thermoregulatory system and metabolism of animals exposed to DDE. Organochlorines, such as DDT, can bioaccumulate and are therefore particularly problematic for top predators. Furthermore, many crocodilians live in regions of the world where DDT continues to be used to

combat malaria or in areas where the breakdown products of DDT, DDE and DDD (1,1-Bis(p-chlorophenyl)-2,2-dichloroethane), remain in the ecosystem. Numerous aspects of poikilotherm metabolism are affected by environmental temperature (Rome, 1990;; Logue et al., 2000; Somero, 2004; Guschina and Harwood, 2006; Bicego et al., 2007). Therefore it is important to know if standard metabolism or preferred body temperatures are perturbed by DDE exposure because these changes may affect the overall health of animals, their reproductive success, and the health and growth rates of hatchlings.

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CHAPTER 2

"RED LIGHT, GREEN LIGHT" A VISUAL REVERSAL STUDY EXPLORING LEARNING AND MEMORY IN JUVENILE AMERICAN ALLIGATORS

Abstract

Learning abilities of juvenile American alligators (*Alligator mississippiensis)* were studied by measuring performance in a serial reversal experiment under controlled laboratory conditions. Ten juvenile American alligators were trained to push a colored Plexiglas target. Once captured, this behavior was reinforced with a food reward. After this behavior was established as a stable and reliable pattern, it was utilized in a learning problem. Alligators were tasked to discriminate between two visually distinct stimuli. The positive stimulus was rewarded, while a response to the negative stimulus was neither rewarded nor punished. Upon reaching a pre-Determined criterion, the signs of the discriminanda were reversed in a series of additional trials. A clear trend of decreasing errors per reversal was observed, indicating mastery of the learning task and behavioral flexibility when utilizing an acquired physical task. We

conclude that juvenile alligators will train successfully to perform a learned visual discrimination task when offered a food reward.

Introduction

Scientists and the general public have always been interested in how different species learn and what anatomical and physiological attributes correspond to learning differences or similarities. Unfortunately, there is no adequate way of ordering species in terms of “intelligence,” simply because, to date no one benchmark can be relied upon to measure or represent an index for intelligence (Bitterman, 1965a). Attempts to discover such a benchmark trait have failed, most likely because such studies have relied on simple memory tasks that are unable to elucidate details of problem solving and any phylogenetic hierarchies (Bitterman, 1965a). However, more complex dynamic learning tasks (i.e. serial reversal problems) can provide insights when the performance of different species is compared (Bitterman, 1965b).

A common method used to assess the ability of an animal to learn is to train the individual to perform a task and then repeatedly change the task that is required of the animal. Such repeated changes allow one to observe how quickly the animal learns a new behavior. A habit-reversal experiment is one such assay, as it relies on the same basic methodology (Bitterman, 1965b). In a habit-reversal experiment animals are presented with two stimuli that are either visually or spatially distinguishable. One of the two stimuli is assigned to be the “correct” choice and consistently produces a food reward when selected by the animal. Visual problems reward the correct stimulus regardless of position. Spatial

problems reward a correct location regardless of what stimuli are present at the location. Throughout the experiment, animals are rewarded for choosing the pre-determined “correct” choice. Once the animal reaches a predetermined criterion of correct choices, the discriminanda are reversed and the negative stimulus is now given a positive sign and rewarded while the previously positive stimulus is now given a negative sign and if selected no longer produces a reward. Experimenters collect data on the total number of errors committed during each reversal. Animals of various taxa, including pigeons, some fish species, turtles, chickens, and rats show progressive improvement and a decrease in the number of errors committed during each reversal (Gatling, 1951; Reid, 1957; Wodinsky and Bitterman, 1957; Bitterman et al., 1958; Warren et al., 1960; Eskin and Bitterman, 1961; Gonzalez et al., 1964; Stearns and Bitterman, 1965; Setterington and Bishop, 1967; Mackintosh and Cauty, 1971). The typical trend is an initial mastery of the problem, a dramatic increase in errors during early reversals and a steady improvement with additional reversals. Errors may increase during early-reversals, as animals tend to persist in selecting the stimuli that previously produced a reward, but as the animal’s experience continues, its selection habit becomes more flexible (Bitterman, 1965b). However, the results in fishes are complicated because studies of a number of species have failed to show progressive improvement while other species show improvement (Wodinsky and Bitterman, 1957; Bitterman et al., 1958; Warren, 1960; Behrend et al., 1965; Behrend and Bitterman, 1967; Setterington and Bishop, 1967; Mackintosh and Cauty, 1971). These contradictory studies in fishes may imply

that the progressive improvement observed in some fish species only appears in fishes under a narrow range of restrictive experimental or reward conditions (Engelhardt et al., 1973).

Crocodilians are interesting organisms for studying learning and memory because of their phylogenetic relationship with birds; a group well characterized for learning and memory (Bitterman, 1965b). The crocodilians appeared in the late Cretaceous Period and they are the sole surviving lineage of a prominent clade of archosaurs known as the Crurotarsi (Brusatte, 2009). The Crurotarsi radiated widely in the Early and Middle Triassic and contained many morphologically diverse forms, such as the armored herbivorous aetosaurs, the ostrich-like *Effigia* and *Shuvosaurus*, large terrestrial carnivorous forms such as the "rauisuchians," as well as semi-aquatic crocodile-like forms such as the phytosaurs. The sister lineage of the *Crurotarsi*, the *Avemetatarsalia*, also radiated widely in the Triassic and includes the remarkable number of morphologically diverse and successful animals such as pterosaurs and dinosaurs. Thus, an investigation of the learning ability of modern crocodilians and their specialized brain function can be compared to the learning capacity and brain function in their sister taxon, birds, to provide clues about learning capacities in the basal archosaur order and about how these capacities changed as the lineage radiated.

Direct fossil evidence of learning capacities is extremely rare and should be substantiated with a thorough understanding of the capacities of the extant lineages. For example, studies of an endocast of an allosaurus brain suggest that

the neuroanatomy of this lineage of dinosaurs was more similar to that of modern crocodilians than to their close relatives the avian dinosaurs (Rogers, 1999). A major difference between the brains of modern birds and crocodilians is that birds have greatly reduced olfactory organs and an enlarged telencephalon, which is suggested to impart greater neuronal complexity such as a capacity for somatosensory processing, whereas allosaurus retained the large olfactory organs and small forebrain (Rogers, 1999). The expanded forebrain is suggested to have enabled birds to have more plastic behavior and thus, if allosaurus had complex behaviors, these behaviors would have been highly structured and not especially flexible (Rogers, 1998). However, although crocodilians have a small telencephalon relative to hindbrain structures, it is not fully known how plastic or structured the behavior of crocodilians is. Crocodilians display a repertoire of complex vocal and behavioral communication signals (Modha, 1967; Garrick and Lang, 1977; Garrick et al., 1978). Both vocal and behavioral cues are used during sexual competition, territory establishment, mate selection and copulation (Garrick and Lang, 1977). This complex social interaction may select for flexible learning abilities. Furthermore, female alligators show nest fidelity from year to year, returning to the same nest site at the beginning of each reproductive cycle (Elsey et al., 2008), which may require the females to learn where the good sites are located and then remain faithful to them.

Very little is known about the ability of crocodilians to participate in learning tasks. The spatial discrimination ability of the spectacled *Caiman* (*Caiman crocodiles)* has been explored (Williams, 1967; Williams, 1968;

Northcutt and Heath, 1971). While details of these studies differed slightly, the basic design was constant and utilized a modified T-maze to determine the ability of the spectacled *Caiman* to reverse a habit formed in a spatial problem. Williams (1967 and 1968) and Northcutt and Heath (1971) observed a decrease in the number of trials required for *Caimans* to learn a new arm after each reversal. Williams (1968) explored the dominance of spatial versus visual cues with spectacled *Caimans*. Williams determined that when *Caimans* were trained to respond to a spatial cue and then presented with a choice point where visual and spatial cues were both available, animals tended to persist in a habit based on the spatial cues instead of incorporating the use of visual cues into their performance in the spatial learning task (Williams, 1967).

Gossette and Hombach (1969) compared the spatial ability of *Alligator mississippiensis* to *Crocodylus acutus*. Both species were asked to discriminate based on location and both species showed progressive improvement, although the sample size used in this study (N=4) was small. Furthermore, Davidson studied the spatial discrimination ability of American alligators utilizing a spatial T-maze and the animals were motivated by escape from heat (Davidson, 1966). The floor of a metal T-maze was heated to 40.56°C, a temperature exceeding the upper lethal temperature of 38°C of alligators (Colbert et al., 1946). Although core body temperature was not measured, it is possible this protocol severely stressed and adversely affected the performance of animals in a learning task. Another potential problem with the Davidson study is that internal body temperature could affect the performance of these animals in such a task (Heath,

1965). The current work explores the ability of juvenile American alligators to perform a visual, rather than spatial, discrimination task when tested without the use of heat or other punishments. To our knowledge, the visual discrimination ability of the American alligator has not previously been studied.

Materials and Methods

All animals were part of a single clutch procured from the Rockefeller Wildlife Preserve, Grand Chenier LA., at approximately 3 weeks of age in September 2006. Animals were housed in plastic tanks, containing an equal depth of water as the reversal apparatus. Previous to reversal training, heaters were introduced into animal tanks to maintain water temperature between 30°C 5°C. Tanks were housed in an environmentally controlled room with an air temperature ranging from 25 °C to 28° C. At 2 years of age, seven Alligators from this clutch were randomly divided into two groups, one group of four individuals and one group of three individuals (Groups A and Group B respectively). Group A and B began reversal training on September 8th, 2008 and October 10th, 2008 respectively. Group A was trained to respond positively to the color red during Reversal 0 while Group B was trained to respond positively to the color green during Reversal 0. These individuals ranged in size from 1.7 kg to 1.30 kg and were trained to perform a simple task when presented with two visually distinguishable discriminanda in order to receive a food reward.

The reversal apparatus was based on the design Bitterman (1965a) used to explore the visual discrimination ability of *Tilapia macrocephala*, with modifications making it more appropriate for the modality of the alligators. The

reversal apparatus (Figure 2.1) consisted of a 75-gallon glass aquarium divided into a start box and a choice point. At the choice point two Plexiglas targets were present, each target had been outfitted with a Tricolor- LED and could be illuminated with one of three different colors. Each target was attached to a single magnetic reed switch. By pushing the target with its nose, the alligator tripped the magnetic switch and completed a circuit causing an automatic feeder to distribute a food reward. The food reward was distributed in the back of the start box, not near the targets. The aquarium was filled with water to a depth of 12.7 cm and maintained at a temperature of 32°C 5°C by a 250 Watt submersible glass aquarium heater.

Reversal training was divided into three progressive steps, each step representing a more difficult task, culminating in reversal-learning and data collection. The goals of the first step of training were to introduce study animals to the experimental routine and to train them to come to expect food only when inside the glass habit-reversal aquarium and nowhere else. During this first step of training, a single individual was selected at random and placed in the glass habit-reversal aquarium. During step one, each Plexiglas target was illuminated with the color blue, a neutral stimulus. This individual was then given 2 Mazuri brand commercial alligator chow pellets at a time until ten pellets were consumed or an hour had elapsed. Individuals were only fed what they consumed in the glass aquarium. This was repeated 3 days a week until the alligator was consuming 10 pellets within the hour. Once that criterion was met, the second step of training began during the next scheduled day of training.

In step two, as in step one, animal housing and handling procedures were kept consistent. Each target continued to be illuminated with the neutral stimulus. However, now each target was baited with a single pellet of food. Each alligator was given a half-hour to consume 10 pellets. If 10 pellets had not been consumed within 30 minutes the alligator was removed from the glass aquarium and returned to the home cage. The experimenter checked on the animal every 5 minutes, if a pellet had been consumed, the experimenter reset the trial by encouraging the alligator to walk back to the start box, opaque dividers were then put in place, the target was baited with an additional pellet of food, and the experimenter replaced the aquarium lid and removed the dividers. This continued until the individual had met the criterion of consuming 10 pellets in a half-hour. Once that criterion was met, the third step of training began during the next scheduled day of training.

The goal for the third step of training was to train the alligators to actually push the Plexiglas targets. When actively searching for food, juvenile alligators move their heads from side to side. If food is not easily found, this movement becomes more aggressive as the individual appears to become frustrated. By training them to expect food to be present in front of the targets, we had hoped that this exploratory behavior could be captured and utilized to push the targets. Just as before, both targets were illuminated with the neutral stimulus. Once in the tank individuals immediately approached the target. As they discovered food was not easily found, the alligators began actively searching for food, which eventually became forceful enough to displace the Plexiglas target. The

experimenter rewarded the individual in the start box. A single press of target defined a trial. After each trial the experimenter reset the apparatus just as in step two. After a few repetitions it appeared the alligator began to associate a food reward with the action of pushing the target. During step two both targets were baited in an attempt to train the alligators to come to expect food from both targets. In step three, both targets were rewarded for the same reason, the training in step two carried over into step three. Alligators showed very little spatial preference and actively explored and engaged both the left and right target with equal frequency. A single push of a target was defined as a trial. Once the criterion of completing ten trials in a half hour was met, data collection began during the next scheduled day of training.

In step three of the training, and during data collection, when the target was pushed an automatic feeding apparatus was briefly activated. An LED and magnetic switch on each target was wired to a circuit board. An Arduino (Ivrea, Italy) processing chip installed on the circuit board allowed the LED and magnetic switch to communicate with a computer. An automated data collection program was written using Python software. This program was used during each trial and recorded which magnetic switch was activated, left or right, and what color the LED was projecting and when the switch was activated. During reversals, when the LEDs were illuminated with red or green, the computer randomly selected which color would be located at the left and right target with the same color never appearing in the same location more than three times in a row. Data collection was also automated in the same manner.

After completing the training protocol, reversal learning began on the next scheduled day. Individuals from Group A were trained to respond to the color red, while Group B was trained to respond to the color green during reversal learning. During reversal learning each individual was given a total of 10 trials, 3 days a week, resulting in a total of 30 trials a week. At the beginning of each 10 trial-set, an animal was placed in the start box of the habit reversal aquarium with the opaque dividers in place. The dividers were then removed, revealing the Plexiglas targets. Throughout the series of visual discrimination tasks, one target would be illuminated with the green LED while the other would be illuminated with the red LED. The box was covered with black upholstery velvet so that the animals could not see the experimenter prior to pushing the target and receiving the reward.

Once the animal had selected the “correct” color 7 out of 10 trials on a single day of data collection, the color of the positive stimulus was reversed. This procedure was repeated until the color of the positive stimulus had been changed ten times. The total number of trials and the total number of errors per reversal was collected for each individual. The total number of errors for each reversal for all seven animals was tested for normality. While some of the reversals contained a normal distribution of errors, not all the reversals were found to contain a normal distribution of errors. For each reversal the mean was calculated of the total errors made by all animals (mean total of errors). This mean total of errors was used to assess the performance of each group in each

reversal. A one-way ANOVA was used to test for a significant difference in the performance between early and later reversals.

Results

Results are plotted for the seven individuals (Figure 2.2) who completed ten reversals (Reversal 1-10) plus the original color reversal problem (Reversal 0). No difference was seen in the performance between Group A and Group B, therefore the results of these groups were pooled. The mean total errors decreased as reversal number increased. The difference in mean total errors (Figure 2.2) between early and later reversal was highly statistically significant (P=0.0024). We also saw a decrease in the variation of performance between individuals.

Discussion

Reversal experiments such as these present two problems. Each problem offers unique insights into learning processes and memory formation. The first problem allows an animal to demonstrate an ability to associate a learned response with a food reward. However, with continual reversal of the positive and negative discriminanda, a second problem arises where the animal is allowed to demonstrate an ability to learn and remember that the responses acquired in the first problem must be flexible.

The general performance pattern observed across numerous species in learning reversal tasks is as follows. In an initial reversal (e.g. reversal 0), a certain number of errors are made, and then in successive reversals (e.g.

reversal 1-3) errors increase to a maximum value. After this peak, errors begin to decrease and eventually plateau. Once this maximum is reached, the rate of decrease in the number of errors with successive reversals differs among species. This rate was quantitatively compared by finding the full width (number of reversals) at half the difference between the initial number of errors and the maximal number of errors of this waveform (FWHM). Figure 2.3 illustrates this methodology. The values for species other than alligators were approximated from published graphs. The FWHM measurement allows comparison of the shape of the performance function and quantitatively represents how quickly each species performance in a learning task improved. In rats (Gatling, 1951) animals returned to the FWHM at reversal 2, pigeons (Reid, 1957) returned at reversal 5, chickens (Bacon et al., 1962) returned at reversal 3. Alligators in the current study returned to the FWHM at reversal 2. Therefore, the performance of juvenile American alligators in a visual discrimination task is similar to that observed in other species including mammals and birds.

The overarching objective of this work was to investigate learning abilities in juvenile American alligators reared in the laboratory. Specifically, the goals of this research were one, to establish the optimal conditions for training and reversal using a food reward, two, ascertain the visual discrimination ability of the American alligator and three, determine the degree of complex learning by testing the flexibility of a learned response using a serial reversal approach. Very little is known about the ability of American alligators to participate in these types of tasks. In order to close this gap, fundamental studies are required to establish

the baseline performance. We believe that a study of the ability of juvenile lab reared American alligators to utilize an acquired physical response in novel situations, such as those presented in serial reversal experiments, will provide insight into the basal learning in Archosaurs.

Reversal problems, such as the one utilized in the current study, are a valuable paradigm for evaluating and comparing the learning ability of different species (Stettner et al., 1967). Furthermore, performance differences of species in these tasks can lend insight into phylogenic differences in behavior and learning ability (Stettner et al., 1967). Birds are the sister group to crocodilians and by comparing the learning ability of these two groups in reversal problems we may gain insight into basal learning in Archosaurs. A variety of bird species, that represent several orders, have all been shown to exhibit a decreasing number of total errors in a series of visual discrimination problems. Chickens (*Gallus gallus domesticus*), pigeons (*Columba livia domestica*), crows (*Corvus americanus)*, all showed progressive improvement in the number of errors committed during a series of visual discrimination tasks. However, quail (*Colinus virginianus)* do not show progressive improvement in the number of errors committed in a serial reversal experiment and therefore do not reflect the pattern seen in other bird species. Alligators show a pattern of decreasing errors in a series of successive visual discrimination problems, the pattern observed in alligators is similar to the pattern seen in many bird species including Corvids. This shared pattern may lend insight into the role of behavioral flexibility and the evolution of learning in basal Archosaurs.

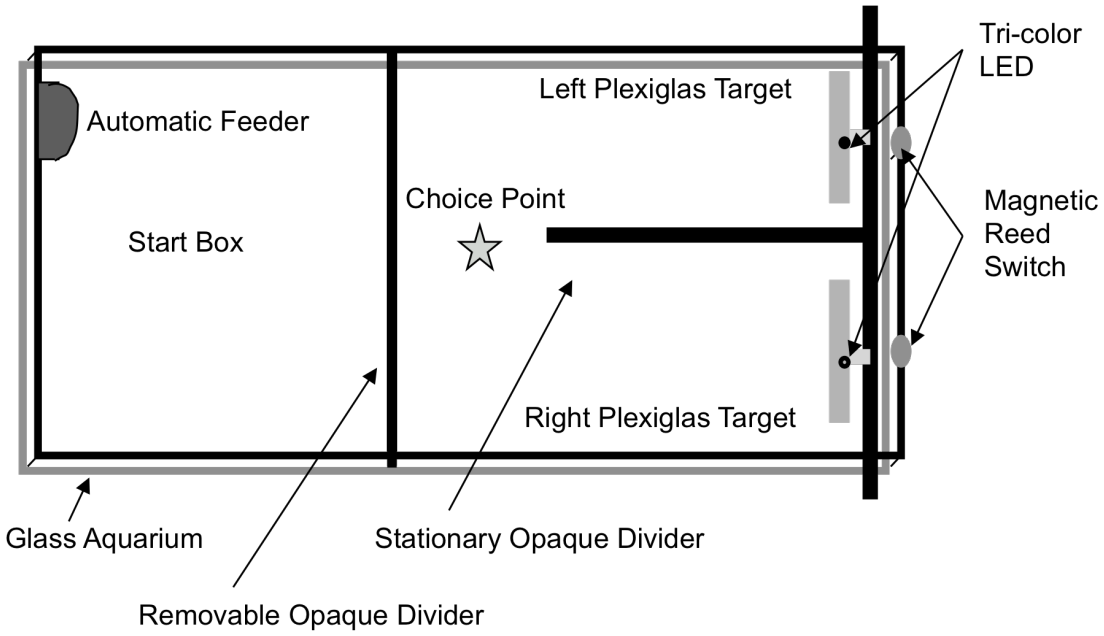

Figure 2.1. Arial view of visual reversal apparatus

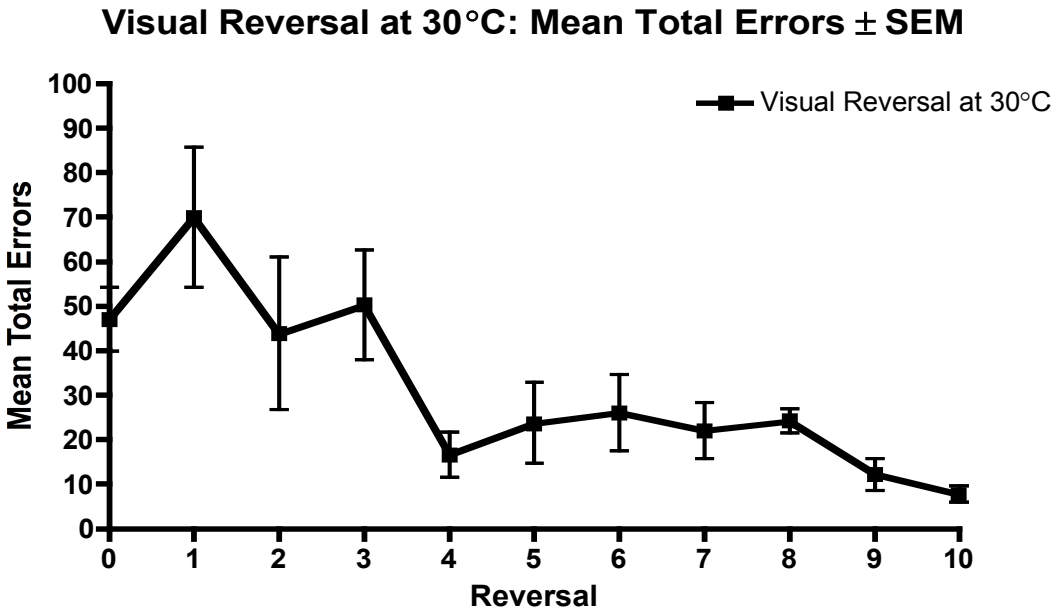

Figure 2.2. Mean total errors per reversal with SEM

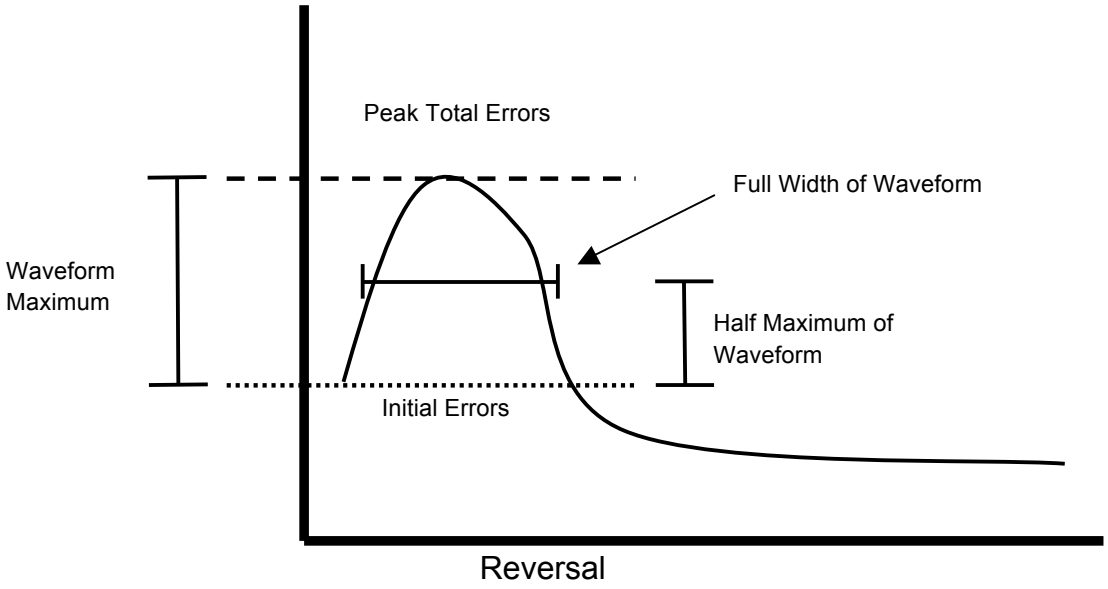

Figure 2.3. Full width (number of reversals) at half the difference between the initial number of errors and the maximal number of errors

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CHAPTER 3

THE EFFECT OF TEMPERATURE ON REVERSAL LEARNING IN JUVENILE AMERICAN ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS)*

Abstract

Ectotherms are sensitive to environmental temperatures. Aspects of poikilotherm metabolism, sensory and motor neuron physiology and the action potentials of muscles have all been shown to be affected by environmental temperature. Recognizing that neuronal physiology is temperature sensitive prompted the question of whether learning and memory are also temperature sensitive. American alligators (*Alligator mississippiensis*) are capable of performing both visual (Araneo and Farmer, unpubl.) and spatial discrimination tasks (Gossette and Homback, 1969). However, it is not known how temperature affects their ability to learn or perform in such discrimination tasks. The effect of temperature on spatial learning in juvenile American alligators was investigated. Eight naive alligators were divided between two temperature regimes. Group A completed a series of 10 habit reversals at 32ϒC and then another set at 22ϒC.

Group B completed a series of 10 habit reversals at 22ϒC and then 10 more at 32ϒC. Performance was measured using mean total error per reversal. A clear trend of decreasing errors was observed. In addition, alligators made fewer total errors at 22ϒC than at 32ϒC regardless of temperature regime. We conclude that alligators perform better in a spatial learning task at 22ϒC than they do at 32ϒC.

Introduction

Studies in learning are aided by development of specialized methods to classify and compare cognitive abilities in several species (Bitterman, 1965). Investigators around the globe have spent time developing techniques and methods to reliably test the adaptability of learning behavior in many species. However, development of methods to test learning in reptiles, especially crocodilian species, has been limited. Large reptiles such as alligators offer the opportunity to study a species capable of adaptation to climatic change throughout millions of years of evolution. Furthermore, crocodilians represent one of two remaining Archosaur lineages. An investigation of its learning ability and specialized brain function may provide clues about basal learning in the Archosaur order. The current research investigated the resilience or susceptibility to temperature changes, of a specialized form of learning in the American alligator.

Temperature has been found to affect synaptic transmission, postsynaptic transmission, postsynaptic integration, spike initiation and conduction of neurons

(Montgomery and Macdonald, 1990). Murray et al. (2007) found that the phospholipid composition of membranes can be affected by temperature in *C.elegans*. Many of these neuron changes could be the result of a change in membrane fluidity similar to that found in *C.elegans*. Furthermore, Somero (2004) investigated the effect of temperature on protein and again found a positive correlation, meaning protein activity increased with temperature. Brain activity is based on neuron activity that has been shown to be affected by temperature (Montgomery and Macdonald, 1990). Therefore, all these temperature sensitive physiological parameters may play a role in learning and memory.

Temperature effects various aspects of learning in a variety of species (Roussel et al., 1982). The effects of temperature on the performance ability in rodents is not clear. Even with extensive training sessions, both hypothermia and hyperthermia perturb memory acquisition (Roussel et al., 1982). A severe learning deficit can be produced in mice with even a moderate degree of hypothermia (Essman and Sudak, 1962; Sudak and Essman, 1962; Sudak and Essman 1963). Contrasting results indicate that mice were still able to learn a brightness discrimination task with a rectal temperature of 27 or 20 °C, but latency increased due to impaired motor activity (Boyd and Caul, 1979). Others have found that hypothermic rats, with a core body temperature decrease of 3-6°C, were still able to learn a memory task when heat was used as a reward (Weiss and Laties, 1961; Panuska and Popovic, 1963). Furthermore, in rats an observed rectal temperature increase of as little as 2 or 3°C can cause amnesia

with the severity of symptoms being related to the magnitude of hyperthermia (Misanin et al. 1979). Roussel et al. (1982) observed that when mice were exposed to extreme temperatures (10 °C or 34 °C) prior to learning an avoidance task, these extreme temperatures greatly reduced a subject's learning ability (Roussel et al., 1982).

Ectotherms are sensitive to environmental temperatures. Aspects of poikilotherm metabolism, sensory and motor neuron physiology, and the action potentials of muscles are affected by temperature (Krekorian et al., 1968; Zerbolio, 1973; Reeves, 1977; Stephens et al., 1982; French, 1985; Montgomery and Macdonald, 1990; Rome, 1990; Somero, 1995; Gray, 1998; Hosler, 2000; Hosler et al., 2000; Logue et al., 2000; Somero, 2004; Guschina and Harwood, 2006; Guderley, 2004; Bicego, et al., 2007; Murray et al., 2007). Recognizing that neuronal physiology is temperature sensitive prompted the question of whether learning and memory are also temperature sensitive; the implication being learning and memory would be most efficient within a specific temperature range. However, one could make an argument that the ability to create and store new memories and associations regarding one's environment at any temperature could offer a substantial fitness advantage.

Only a few studies have been done to examine the effect of temperature on memory and learning in poikilotherms (Krekorian et al., 1968; Riege and Cherkin, 1972; Zerbolio, 1973; Borsook et al., 1977; Roussel et al., 1982; Stephens et al., 1982). In Goldfish, Riege and Cherkin (1972) attempted to assess the effects of temperature on memory and learning. At a given shock

level, temperature was found to have an effect on avoidance scores, with avoidance of shock treatments decreasing as temperature decreased (Riege and Cherkin, 1972). However, while memory of punishment was dependent on temperature, it was also found to be dependent on shock level and test interval after shock (Riege and Cherkin, 1972).

Zerbolio (1973) conducted a conditioned avoidance response in Goldfish and found that temperature, days of training and the interaction between the two had an effect on avoidance rates. Zerboilio (1973) found a positive relationship between temperature and avoidance rates, in other words avoidance rates increased with increasing temperature. Borsook et al. (1977) attached floats to the ventral surface of fish and observed the rate of compensation with in three temperature regimes to determine the learning ability of fish. From their results, Borsook et al. (1977) concluded that acclimation temperature influences the learning ability of fish (Borsook et al., 1977).

A performance index was used to determine how much time a fly spent in a non-preferred temperature compared to a preferred temperature, with more reversals each fly spent less time in the negative reinforcing temperature (Zars and Zars, 2006). The study found that avoidance of the higher temperature, in the 24/30, 24/33 and 24/37 temperature pairings, increased as temperature increased. Furthermore, low temperatures were not shown to reinforce place memory as strongly as high temperatures (Zars and Zars, 2006).

Honeybee workers maintain the brood within a narrow temperature range around 34.5 □ 1.5 °C. (Jones et al., 2005). Jones et al. (2005) found that short-

term memory in Honeybees, *Apis mellifera,* was affected by temperature of pupation. However, long-term memory was not affected by temperature of pupal development (Jones et al., 2005). Tautz et al. (2003) and Von Frisch (1993) show that bees reared at 36°C preformed normal communication dances, while bees at 32°C preformed fewer dances and the dances that are preformed tend to be shorter than those of bees that pupated at 36°C. This study investigated the effect of temperature on learning and memory and fluctuating asymmetry, and reared bees within the normal temperature with in a hive as well as temperatures that could be experienced along the hive's margins. Short-term memory and learning was significantly affected by rearing temperatures. Rearing temperature was not found to have an effect on long-term memory (Jones et al. 2005).

Krekorian et al. (1968) found that maze learning is temperature dependent in the desert iguana, *Dipsosaurus dorsalis*. Specifically, they found that animals tested at their preferred body temperature outperformed their contemporaries tested at cooler temperatures. Desert iguanas have a preferred body temperature around 42°C (Krekorian et al., 1968). Twenty lizards were randomly assigned to one of four experimental groups. Heat was used as a reward and was supplied through tactile contact of the lizard's body with the floor of the goal box. Experimental groups all had the same difference in body temperature from goal box, but the body temperature and goal box temperatures were different. Learning curves for the two groups are similar in shape, while not statistically significant, the group that experienced the lower temperatures took longer to run the maze. Lizards closest to their preferred temperature learned faster on two

separate mazes. Furthermore, Krekorian found that more individuals met the learning criterion at 32°C than 27°C (Krekorian et al., 1968).

Many of these studies may be compromised by utilization of a time component, i.e. ,trial length, (Borsook et al., 1977) in their learning criteria, or use of a punishment, such as shock (Reige and Cherkin, 1972) that may be more intense at higher temperatures. Other studies may also be compromised by approaches that can create potential artifacts by the use of temperature as a reward; such gradients can act to differentially reinforce behavior (Krekorian et al., 1968). Where temperatures introduce a stronger reward value, performance values would appear to increase due to the strength of this reinforcement not because memory acquisition is enhanced. In other words, if we accept that animals prefer one temperature to another and that preference is enough of a reward to increase performance values then the temperatures are not actually improving the physiological components of memory. Performance is merely increasing due to the reinforcement (Krekorian et al., 1968).

An alternative and more robust approach to address the question of temperature and memory/learning is the use of reversal studies. These offer a useful method to explore temperature and learning for several reasons. One, the performance of individuals is not based on a time component. Two, alligators have been shown to learn effectively without the use of a punishment. Three, reversal studies do not present the opportunity for differential reinforcement because such studies do not rely upon the use of temperature gradients.

A common method used to assess the ability of an animal to learn is to train the individual to perform a task and then repeatedly change the task that is required of the animal. Such repeated changes allow one to observe how quickly the animal learns a new behavior. A serial-reversal experiment is one such assay, as it relies on the same basic methodology (Bitterman, 1965b). In a serial-reversal experiment animals are presented with two stimuli that are either visually or spatially distinguishable. One of the two stimuli is assigned to be the “correct” choice and consistently produces a food reward when selected by the animal. Visual problems reward the correct stimulus regardless of position. Spatial problems reward a correct location regardless of what stimuli are present at the location. Throughout the experiment, animals are rewarded for choosing the pre-determined “correct” choice. Once the animal reaches a predetermined criterion of correct choices, the discriminanda are reversed and the negative stimulus is now given a positive sign and rewarded while the previously positive stimulus is now given a negative sign and if selected no longer produces a reward. Experimenters collect data on the total number of errors committed during each reversal. Animals of various taxa, including pigeons, some fish species, turtles, chickens, and rats show progressive improvement and a decrease in the number of errors committed during each reversal (Gatling, 1951; Reid, 1957; Wodinsky and Bitterman, 1957; Bitterman et al., 1958; Warren et al., 1960; Eskin and Bitterman, 1961; Gonzalez et al., 1964; Stearns and Bitterman, 1965; Setterington and Bishop, 1967; Mackintosh and Cauty, 1971). The typical trend is an initial mastery of the problem, a dramatic increase in errors during early

reversals and a steady improvement with additional reversals. Errors may increase during early-reversals, as animals tend to persist in selecting the stimuli that previously produced a reward, but as the animal's experience continues, its selection habit becomes more flexible (Bitterman, 1965b).

American alligators are capable of performing both visual and spatial discrimination tasks (Gossette and Homback, 1969). However, it is not known how temperature affects their ability to learn or perform in similar discrimination tasks. One possibility is that performance values decrease, indicating animals are less effective learners at certain temperatures, or do the time components used to measure performance values simply increase, indicating that learning is just as effective but simply takes longer. Specifically, learning may not actually be delayed at specific temperatures (if one could directly measure neuronal association), but rather reflects the delayed muscle activity used to assess learning. This study will attempt to answer the first question and lay groundwork for the second.

Materials and Methods

The activity level of poikilotherms is set by environmental temperature. However, using a food reward means that alligators are motivated by hunger level, which is also influenced by temperature. This level of motivation will also be set by temperature and how quickly they digest. To keep activity levels at an efficient level, the temperature of the home cages was maintained at 30°C. Animals were housed individually in 54-gallon cattle troughs each heated with an

aquarium heater; all cattle troughs were housed in a temperature-controlled room.

All experiments were conducted in an environmentally controlled room with a temperature range of 26-28°C. The experimental apparatus consisted of a 75-gallon aquarium (measuring 1.22 m, 0.47 m wide and 0.79 m deep) filled with approximately 6.35 cm of water at temperature of 30 ± 1.5°Celsius. A 250-Watt aquarium heater was used to maintain the temperature of the water with in the aquarium. The sides of the aquarium were covered with heavy weight black upholstery velvet, limiting the amount of light within the aquarium. All attempts were made to ensure that the only light available during data collection come from the reversal apparatus (see below). A lid for the aquarium was also constructed from the same black velvet. Two black plastic trays were used as a partisan to divide the tank into two regions a start box and a goal box. The goal box was further divided into two alleyways by a quarter in thick pieces of opaque acrylic sheeting. Each alleyway led to a single Plexiglas target.

Each target was constructed from a single half inch thick piece of frosted Plexiglas cut into 11.43 cm by 11.43 cm square. A Radio Shack technology Plus, 5mm High Brightness Full-color LED was then installed in the piece of Plexiglas and silicone was used to keep it in place. A Full-color LED is manufactured to produce three colors, blue (470 nm), red (624 nm) and green (525 nm). This made a convenient light source because a single LED could be used during both training and data collection phases of the experiment. Each target was then equipped with a Guard N.O./N.C. magnetic reed switch. As the target was

depressed by an animal, the switch would come in close proximity to the magnet closing a circuit activating an automatic feeding apparatus. The LED and magnetic switch of each target were wired to a circuit board. An Arduino processing chip installed on the circuit board allowed the LED and magnetic switch to communicate with a computer. The computer monitored and recorded what magnetic switch was activated, left or right, and what color the LED projected when the switch was activated. During reversals, the LEDs were illuminated with green and the computer randomly selected which target, left or right, would produce a food reward. At the end of the first set of reversal a Chi-squared was run on the computer selection to ensure that the computer program was indeed randomly selecting both the left and right targets with the same frequency.

Attempts were made to distribute a food reward using a Fish Mate P21 automatic pond fish feeder that had been modified with a small rotary motor. In previous reversal studies we have found that when the motor was activated, by a correct selection, alligators would walk to the back of the tank to the area where the food reward was delivered. Therefore, even though the fish feeder is not used to distribute a reward the sound of the motor appears to facilitate learning.

Training was divided into three progressive steps, where each step involves a more difficult task, culminating in reversal learning and data collection. Each training step was fully automated by a computer program; the experimenter simply had to select which training protocol was appropriate.

The goal of the first step of training was first to introduce study animals to the experiment's routine and second to train them to come to expect food only when they are in the glass habit reversal aquarium and nowhere else. On training days, all individuals were transferred to a holding container. The holding container was a Rubbermaid container filled with water held at a temperature of 30°C. The holding tank is important because it allows individuals to be kept warm while waiting for other individuals to finish their trails.

During this first step of training a single individual was selected at random, removed from the holding tank and placed in the glass habit reversal aquarium. During step one, each Plexiglas target was illuminated with the color blue, a neutral stimulus. This individual was then given two pellets at a time until 10 pellets were consumed or an hour had past, whichever came first. Individuals were only fed what they consumed in the glass aquarium. This was repeated 3 days a week until the alligator routinely consumed 10 pellets within an hour. Once this criterion was met, the second step of training was begun on the next scheduled day of training.

In step two, each target was illuminated with the neutral stimulus. However, now each target was baited with a single pellet of food. Each alligator was given a half-hour to consume 10 pellets. The experimenter was responsible for checking on the alligator every 5 minutes, if a pellet had been consumed, the experimenter would then reset the trial by encouraging the alligator to walk back to the starting box, the dividers were put in place, the target was baited with an additional pellet of food, the experimenter would then replace the lid and pull the

dividers. This continued until the individual had met the criterion of consuming ten pellets in a half-hour. Once that criterion was met, the third step of training was begun on the next scheduled day of training.

The goal for the third step of training was to train the alligators to actually push the Plexiglas targets. When actively searching for food, juvenile alligators move their rostrum from side to side. If food is not easily found this movement becomes more aggressive as the individual appears to become frustrated. By training them to come to expect food to be present in front of the targets we have been able to capture this exploratory behavior and utilize it for target training. Just as before both targets were illuminated with the neutral stimulus. Once in the tank, individuals immediately approached the targets. As they discovered food is not easily found the alligators began to actively search for food. In previous studies, this searching has become forceful enough to displace the Plexiglas target at which time an experimenter rewarded the individual. A single press of a target defined a trial. After each trial the experimenter reset the apparatus just as in step two. In previous experiments the alligator began to associate a food reward with the action of pushing the target after only a few repetitions. During step two both targets were baited in an attempt to train the alligators to come to expect food from both targets. In step three, both targets were rewarded for the same reason. Alligators showed very little spatial preference and actively explored and pressed the left and right target with equal frequency. Once the criterion of completing 10 trials in a half-hour was met, data collection began during the next scheduled day of training.

Once an individual had completed each step to criterion, data collection was begun. During reversal learning each individual was given a total of 10 trials per day, 3 days per week resulting in a total of 30 trials in a 7-day interval. Group A completed a series of ten habit reversals at 32ϒC and then another set at 22ϒC. Group B completed a series of 10 habit reversals at 22ϒC and then 10 more at 32ϒC. Group A was trained with the right target as the positive discriminanda while Group B was trained to respond positively to the left target. For each consecutive reversal, individuals were reversed, on the next scheduled day, once an individual achieved a performance of 7 correct trials out of 10 trials. This was repeated for a total of 10 reversals. The temperature régimes were then switched and a second set of 10 reversals was completed. Previous studies have found individual variation in the performance values of animals in a learning task, with some individuals excelling while others were never able to meet criteria even on an initial reversal (Araneo and Farmer, Fig. 2.2, unpubl.). Therefore, a comparison between groups may not be effective. Instead it may be more effective to compare the performance during an initial set of reversals to the performance of those same individuals in an additional set of reversals after a new temperature regime is established.

For the group of animals completing reversals at 22°C an additional procedure was utilized to ensure that the animals would be completing reversals at 22°C. Prior to the completion of the third step of the training protocol, the smallest and the largest animals were placed in a holding tank containing water

at a temperature of 22°C. The cloacal temperature of these individuals was then taken every 10 minutes. The smallest individual reached a temperature of 22°C after 20 minutes, while the largest individual reached a temperature of 22°C after 45 minutes. It was determined that it would take an average of 30 minutes for the majority of the animals to reach a temperature of 22°C. Every animal was then placed in the holding tank one at a time for 30 minutes to ensure enough time for their cloacal temperature to reach 22°C. Therefore, on each day of data collection, were an animal would be completing trials at 22°C, the animal was removed from its home cage and placed in the holding tank for 30 minutes prior to data collection.

The experiment began by establishing a baseline ability of alligators in a spatial problem. With these baselines established, we repeated the experiment with the same individuals completing an additional set of reversals in a second temperature regime. Such an experimental setup allowed for a comparison between the results of the two temperature experiments. Performance was measured using mean total error per reversal. A comparison was achieved by evaluating the mean total errors per reversal at the two different temperature regimes.

However, two additional techniques were also utilized as a way to gauge temperature effects on learning and memory. One, a linear regression was utilized in order to shine light on the relationship between days spent in each reversal and the number of successful trials. Two, logistical regressions were used to look at the relationship between covariant values. This spatial experiment

has a number of variants. For analysis of these variants we broke the data into “yes” and “no”. In this way we were able to look at the effect (if any) the variant has on the outcome individually. By utilizing logistical regressions we were able to look at the following variants: days spent in each individual reversal, reversal number and temperature. Logistical regressions produce an estimate coefficient or coefficient value. Furthermore, each coefficient value can have a positive or negative sign. This value is a measure of the relationship between the variant and the outcome. A higher number on the graph represents a higher correlation between the variant and the outcome, in our cases successful trials.

Results

American alligators can learn a physical task when training is associated with a positive reward. Statistical values, of number of successful trials, taken over all alligators are significant and show an improvement in the number of successes achieved by each alligator as days within any given reversal increases (Figure 3.1, $p=0.0003$). As the days progress within a reversal, animals performed more successfully and therefore made fewer errors. A linear regression of number of successes per days in any reversal standardizes the day of each reversal. The grey line includes all trials. The black line only takes into account those days were the animals completed all ten trials. The darkness of the circles represent the number of times that value occurred, with grey circles the value occurred less often and black circles indicating the value occurred far more frequently. When one looks at the relationship between days spent in each

reversal and the number of success a clear trend of decreasing errors per day in reversal is observed (Figure 3.1).

Temperature was also found to have an effect on the spatial learning ability of juvenile American alligators. There was a statistical significance between the number of successful trials at the lower temperature (22°C), p=0.0282, when compared to the number of success at the higher temperature (32°C) when the performance of every animal across every reversal is taken into account. In addition, alligators made fewer total errors at 22ϒC than at 32ϒC regardless of when they experienced each temperature (Figure 3.2). By utilizing Logistical regressions we looked at the following variants: days in each individual reversal (Figure 3.3), reversal number (Figure 3.4) and temperature (Figure 3.5). Logistical regressions produce an estimate coefficient or coefficient value. Additionally, as the number of days within each reversal increased all alligators made fewer errors (Figure 3.3), based on the observation that none of the coefficient values are negative. The coefficient value of alligators numbered 22, 11 and 24 is very small, indicating these alligators showed little improvement. The greatest improvement, and highest coefficient value, was seen in the alligators numbered 12, 1 and 14, while alligators numbered 5 and 6 represent intermediate coefficient values. After running a Logistical regression for the reversal number variant, we see a positive relationship between reversal number and number of successful trials for alligators numbered 11, 12, 1, 24 and 14. A negative coefficient value is seen for alligators numbered 22, 5 and 6. However,

none of these negative values are as large as the positive coefficient values of alligators numbered 11, 12 and 14.

Finally, a logistical regression of the temperature variant was also run. Here 32°C was assigned a positive value while 22°C was assigned a negative value. Alligators numbered 22, 5,1, 24 and 14 all show a strong relationship between the lower temperature of 22°C and the number of successful trials. Three of the individuals (alligators numbered 11, 12 and 6) show a relationship between 32°C and the number of successful trials. However, overall a significant effect was observed towards 22°C. We conclude that alligators perform better in a spatial learning task at 22ϒC than they do at 32ϒC, however the performance of individuals is highly variable.

Discussion

The effect of temperature on spatial learning in juvenile American alligators was investigated. Temperature affects a variety of neuronal processes both in endotherms and ectotherms. However, whether temperature is a significant environmental modifier of the ability of crocodilians to master these problems was unknown and not well tested. The results of our research indicate that American alligators perform better in a spatial discrimination task at 22 °C than they do at 32 °C. A subject's ability to discriminate between two stimuli based upon positional or spatial cues is a spatial experiment. When the spatial cues are presented in a series of reversals, it is called a spatial reversal experiment and our approach tasked Alligators to perform two series of ten

reversals each. The reversals embody several learning problems, the first learning to associate a specific physical task with a reward and the second learning an association between a specific location and a reward. Finally, once the stimulus signs are reversed an animal must master an additional problem of learning and demonstrating successful flexible responses - to different stimuli.

Aspects of ectotherm physiology are particularly sensitive to environmental temperature, including the cognitive abilities of ectotherms (Krekorian et al., 1968; Zerbolio, 1973; Borsook et al., 1977; Zars and Zars, 2006). The relationship between temperature and cognitive performance has been investigated in the laboratory with the common prediction that the higher the temperature, the better the performance. Similarly, we predicted the performance of juvenile American alligators in a spatial learning task would be enhanced at higher temperatures due to the greater activity of proteins and overall increased metabolic rate observed in poikilotherms at higher temperatures. Such results would resemble those seen in the desert iguana, were we would anticipate the best results for alligators to occur at 32°C, the temperature closest to the preferred body temperature of American alligators (Krekorian et al., 1968; Farmer, 2008).

Our results showed enhanced performance occurred at the cooler experimental temperature of 22°C. French (1985) saw that the performance of fish was enhanced when animals were tested at a temperature lower than the temperature experienced during memory formation. In our experiment we saw that overall both Group A and B made fewer errors at 22°C. If our animals

followed the pattern seen by French, only the group originally trained at 32°C would have preformed better at 22°C. Therefore, the temperature our animals experienced during memory acquisition did not affect memory utilization because we saw that individuals from both temperature treatments made fewer errors at 22°C. Additionally, in fish individuals reach an upper limit where higher temperatures are found to be detrimental to learning. Therefore we can say that memory formation and utilization are modulated by temperature and that temperature extremes can perturb functional memory and learning processes. Alligators prefer a temperature of 30°C after feeding (Farmer et al., 2008). Therefore, 32°C may represent an upper limit of functional memory formation accounting for the increased number of errors at higher temperatures. However, due to the temperature extremes these organisms endure in their natural habitat, we did not expect 32°C to be detrimental to learning.

Thirty-two degrees C is a temperature that these animals potentially experience on a daily basis. Furthermore, the thermal maximum of this species is 38°C. Therefore, it is unlikely that 32°C is going to be detrimental to learning. A far more likely, but unconventional, explanation for a better performance at a cooler temperature surrounds the alligator's stress and hunger level. Because it is unlikely the animals in the current experiment reached the same satiation point that Farmer's animals reached, it is possible that the animal's hunger level was responsible for setting up behavioral patterns that account for the increased performance at the cooler temperature. Based on the laboratory results seen by Farmer et al. (2008), we can predict a similar pattern in wild populations. In the

wild, animals at 30°C, whose hunger is not completely satisfied, may seek out cooler temperatures to reduce the stress of hunger and the temperature-induced metabolic ramp up. We suggest the housing and animal care arrangement was potentially stressing our animals. In other words, because our experiment relied on motivating the animals with food, we assume our animals always experienced a certain level of hunger. Therefore, when the animal was placed in the reversal arena at the warmer temperature the animal's stress level increased, causing the animal to make a greater number of errors. However, when the animal was placed in the reversal apparatus at the cooler temperature, the temperature corresponding to the preferred body temperature for our animal's hunger level, the animal's stress level decreased and they were able to make fewer errors.

While American alligators perform better in a spatial discrimination task at 22 °C than they do at 32 °C, the performance of individuals is highly variable. The variables responsible for this individual variation were explored using logistical regressions. From these regressions it is clear that some animals seemed to be more sensitive to one variable, such as reversal number, while other individuals appeared to be sensitive to another variable such as trial number. In other words, in every individual a relationship between number of success and at least one of the variables we considered was observed. However, the same relationship was not seen across all alligators. For example, alligator 14 showed a strong relationship between days in reversal, reversal number and 22°C. Alligator 22 showed the strongest relationship between reversal number and 22 °C, but not for days in reversal. Therefore, our logistical

regressions demonstrate the individual variation in performance. Additional variables such as incubation temperature and individual responses to hunger stress could also help to explain the variation in performance values we observed.

In honeybees, it is seen that temperature of pupation differentially effects short-term and long-term memory. Rearing temperature has been shown to affect a bee's ability to perform communication dances. Specifically, bees reared at a low temperature perform fewer dances than bees reared at higher temperatures (Jones et al., 2005). Therefore, just as in honeybees, it is possible that the temperature of incubation may be the source of this variation. Incubation temperature is known to affect sex, body size, energy reserves and metabolic rate in American alligators (Allsteadt and Lang, 1995; Western et al., 2000). Incubation temperature may also be affecting neuronal processes that are responsible for learning and memory. Therefore, it is possible that incubation temperature could account for the individual variation we observed in the spatial discrimination task. An alternative explanation that may account for the individual variation we saw in our covariant analysis surrounds an individual alligator's response to hunger stress. Our covariant analysis explored a number of variables that help to quantify an individual's performance in a learning task, in other words each covariant can be thought of as a measure of performance. The stress of being hungry could have had a differential effect on each one of these measurements of performance. For example, the stress of being hungry may affect the relationship between days in reversal and success for one animal and

the stress of being hungry may affect the relationship between reversal number and success for another animal.

American alligators are a useful study organism for cognitive studies because they share certain life history characteristics with birds and mammals. For example all three groups exhibit parental care, social groups and predatory behavior. Furthermore, certain ecological factors such as food type could affect the evolution of cognitive abilities in a similar manner in both ectotherms and endotherms. However, certain ecological variables, like temperature and climate, may have differential effects on neuronal processes in ectotherms and endotherms. The current study explored the effect of environmentally relevant temperatures on the spatial discrimination ability of American alligators. These environmental temperatures are temperatures these animals could potentially experience on a daily basis. However, in this study of this ectothermic species we saw a significant difference in the number of errors committed at the cooler temperature. Therefore, these results indicate that the flexible learning abilities in ectotherms may be influenced by environmental pressure not seen in endotherms. Furthermore, such pressure may imply these abilities are under a different evolutionary pressure as well.

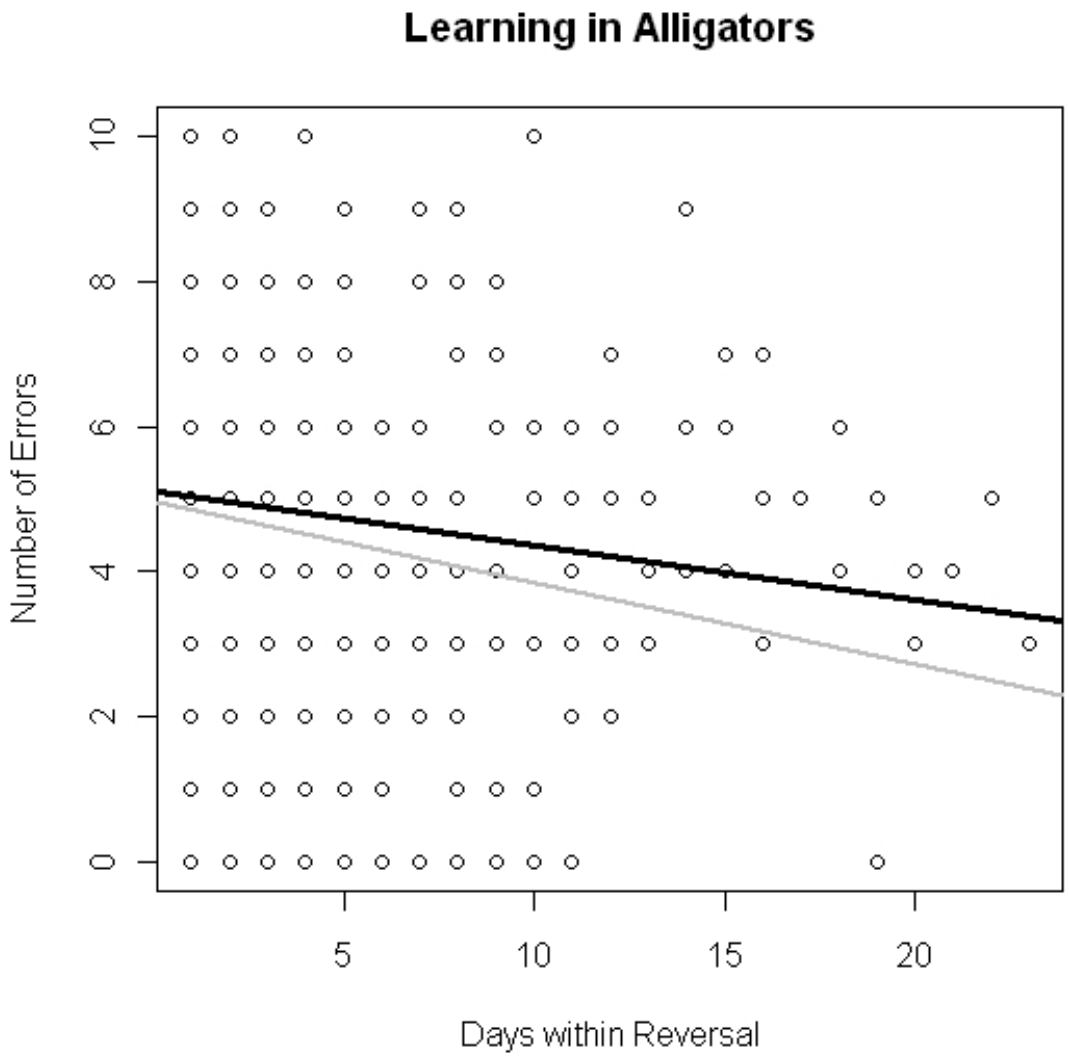

Figure 3.1. Linear regression of spatial temperature data showing a decrease in the number of errors committed by an animal as days within a reversal increased, grey line includes all days regardless of whether all animals completed ten trials, black line includes only those days were all animals completed all ten trials.

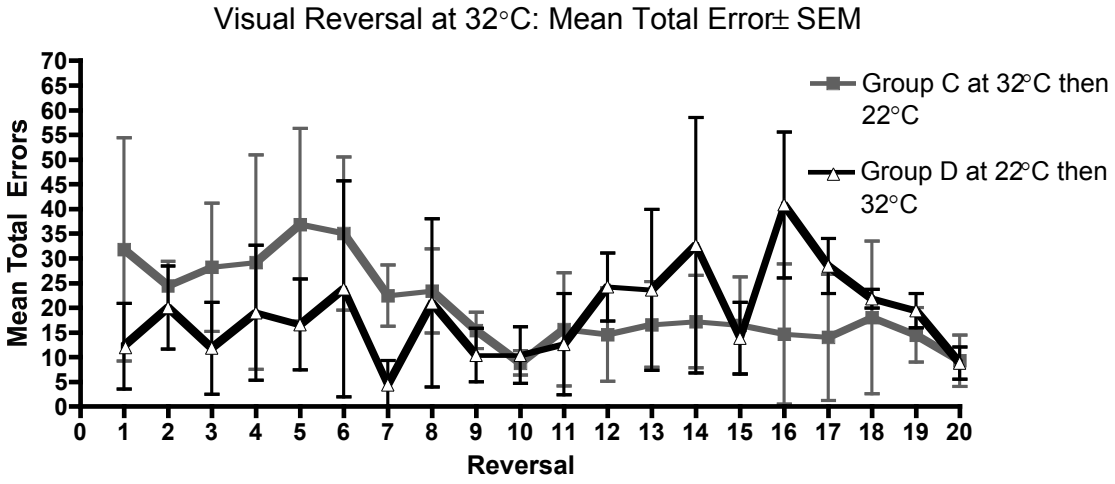

Figure 3.2. Mean total errors per reversal, first set of reversal was run at one temperature, then for the second set of 10 reversal the temperature treatment had been switched, a clear trend of decreasing errors per days spent in each reversal was observed, alligators made fewer total errors at 22ϒC than at 32ϒC regardless of temperature regime (p=0.0282).

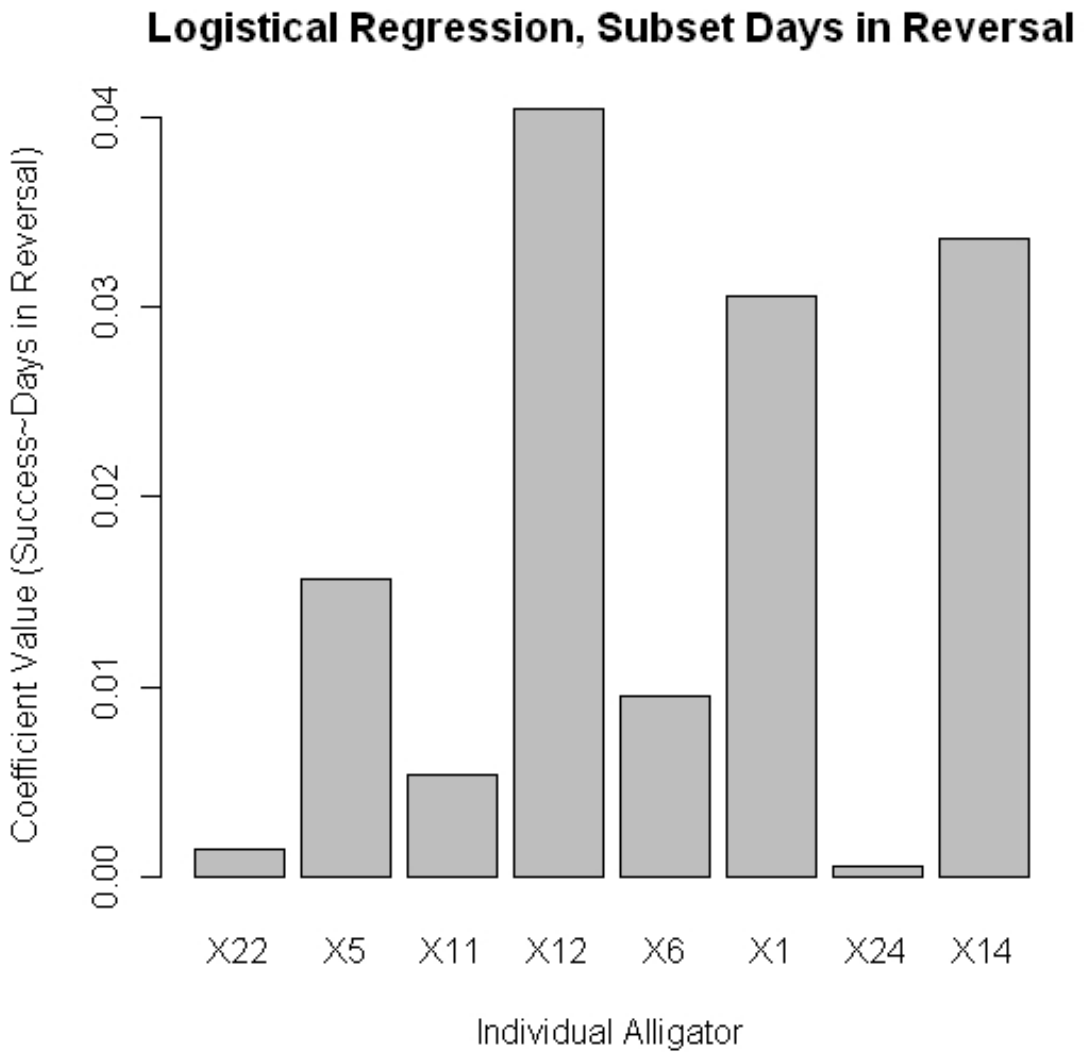

Figure 3.3. Logistic regression analyzing the relationship between days in reversal and successful trials

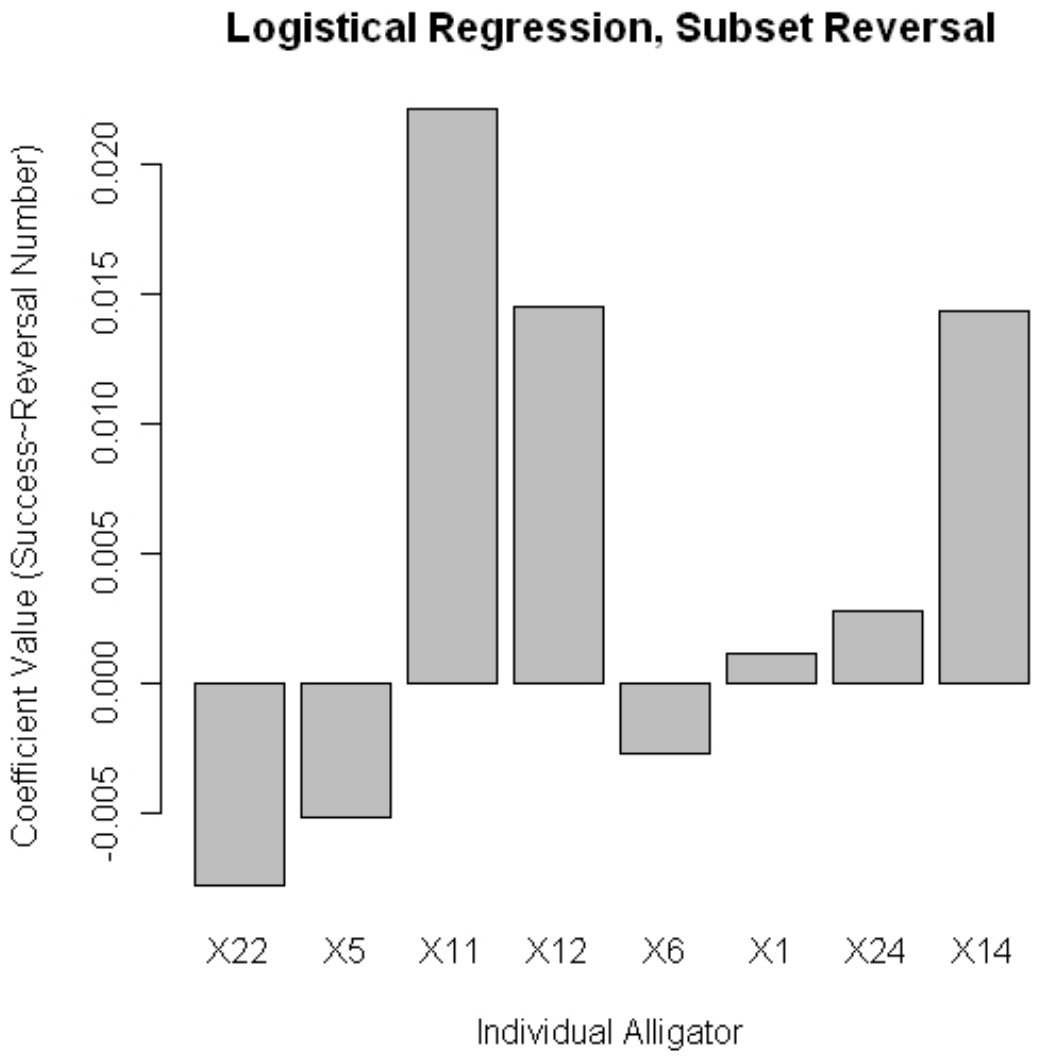

Figure 3.4. Logistic regression analyzing the relationship between reversal number and successful trials

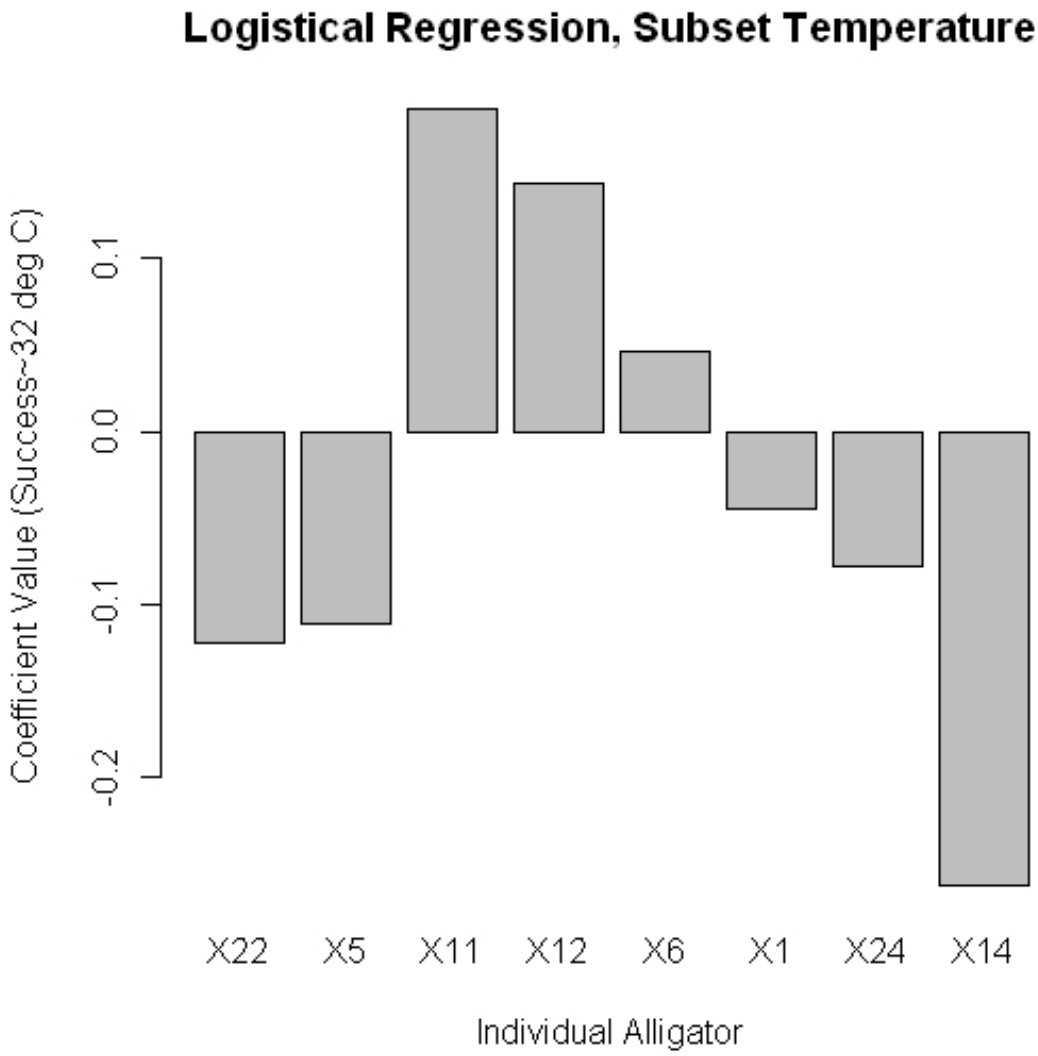

Figure 3.5. Logistic regression analyzing the relationship between temperature and successful trials

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CHAPTER 4

EFFECT OF DDE ON LEARNING IN AMERICAN ALLIGATORS

Abstract

The current study explores the effects of *in ovo* DDE (1,1-dichloro-2, 2-bis[p-chlorophenyl] ethylene) exposure on learning and behavior of hatchling American alligators (*Alligator mississippiensis*). Specifically, we investigated if organochlorines, such as DDE, affect cognition of American alligators by comparing the performance of individuals exposed as embryos to an organochlorine to the performance of control individuals in a spatial learning task. Both field and laboratory evidence show that alligator populations are susceptible to the contaminant DDE. However, little is known about the effects DDE has on the behavior and learning ability of these organisms.

Learning and memory play a crucial role in a variety of essential behaviors such as territory establishment and parental care. In order to address this question, a modified T-maze was used to elucidate the effect of DDE exposure on the learning and memory ability of this species. Hatchling American alligators, exposed to organochlorines, through maternal transfer, made substantially more errors in a learning task than control individuals who had not been exposed to organchlorines. We conclude that DDE affects the acquisition of a learning task.

However, it does not seem to affect the utilization of acquired memory. Because learning and memory are essential elements to these behaviors, if indeed perturbed by organochlorines, exposed populations could suffer a substantial fitness cost detrimentally affecting the dynamics of exposed populations.

Introduction

The worldwide use of the synthetic pesticide DDT (1,1,1-trichloro-2,2,-bis p-chlorophenyl ethane) has exposed both humans and wildlife to this pollutant and it's breakdown products (Kleinow et al., 1987). A variety of morphological, developmental, and physiological abnormalities in both humans and wildlife have been linked to DDT exposure (Schantz and Widholm, 2001). Furthermore, DDT continues to be used to combat malaria in sub-Saharan Africa and other warm, humid regions of the world, and thus many wildlife populations continue to be exposed to DDT (Wu et al., 2000). Numerous adverse effects and behavioral changes have been observed in birds, the sister group to crocodilians exposed to organochlorine compounds.

For example, environmental DDT exposure alters the size of the forebrain and changes the song control system and nuclei necessary for song production and normal sexual behavior in the American robin (*Turdus migratorius*) (Iwaniuk et al., 2006). DDT is lipoophilic and has been linked to deaths in Western grebes *(Aechmophorus occidentalis)* due to accumulation in fatty intracerebral tissue (Dolphin, 1959; Hunt and Bischoff, 1960). DDT and its breakdown products also have estrogenic activity in birds as well and can cause alterations to the sexual characteristics, similar to those seen in alligators (McLachlan, 1993; Kelce et al.,

1995; Jobling et al., 1996). Sex organ development, behavior and fertility are affected by exposure to estrogenic chemicals. For example, male sea gulls living in contaminated ecosystems ignore nesting colonies (Hunt and Hunt, 1977). Furthermore, female sea gulls may pair and nest together (Luoma, 1992). Additionally, when gull eggs are treated with DDT male female sex reversals are produced (Fry and Toone, 1981). Furthermore, abnormal secondary sex characteristics are seen in roosters (*Gallus gallus*) exposed to DDT (Burlington and Lindeman, 1950). Even though it is known that these compounds can derail normal cerebral function in birds and other species, little is known about the effects of organochlorines on the crocodilian brain.

American alligators (*Alligator mississippiensis)* exposed to organochlorine contaminants, including DDE, show multiple reproductive abnormalities, low clutch viability, reduced phallus size, and altered plasma hormone concentrations (Woodward et al. 1993; Guillette et al., 1994, 1996b, 1997, 1999b, 2000; Crain et al., 1998; Pickford et al., 2000). The hepatotoxic and other effects of high levels of exposures to organochlorines are relatively well understood but the effects of lower doses, while not immediately lethal, may impact species through effects on behavior and cognition. Although organochlorines affect behavior and learning in children (Keifer and Mahurin, 1997; Eskenazi, 2006) and other animals (Eriksson et al., 1990), a literature search was unable to turn up any studies that have specifically assessed the effects of these molecules on learning and behavior in crocodilians, where low doses known to exist today may critically impact survival and complex social behaviors. Critical behaviors that may be adversely affected

by the persistence of these contaminants are breeding, nurturing and rearing. Crocodilians exhibit extensive parental care (Hunt 1975; Pooley 1977). Female parental care starts with nest building and nest defense throughout the incubation period (Mcilhenny 1935; Joanen, 1969; Joanen and McNease, 1972; Hunt and Watanabe, 1982). However, parental behavior extends beyond incubation. Crocodilian mothers have been observed scraping open nests to retrieve new hatchlings as well as using their teeth to gently open any un-hatched eggs (Watanabe, 1980). Additionally, females will continue to defend their pods, groups of hatchlings, through the summer and into the following spring (Deitz, 1979). In addition to parental care behavior, crocodilians display a repertoire of vocal cues starting as hatchlings and continuing throughout the life of the adult (Modha, 1967; Garrick and Lang, 1977; Garrick et al., 1978).

The early vocal cues are thought to be critically important to the mother-offspring interaction. After placing a hatchling at a roadside, a researcher observed a mother carrying a vocalizing hatchling back to the nest from which it had been removed (Kushlan, 1973). Pods will remain together for the first year (Woodward et al., 1987) and individuals within the pods will vocalize to each other at the approach of an intruder. Crocodilians display a repertoire of behavioral cues as adults as well (Modha, 1967; Garrick and Lang, 1977; Garrick et al., 1978). Both vocal and behavioral cues are used during sexual competition, territory establishment, mate selection and copulation (Garrick and Lang, 1977). Both male and female crocodilians establish and defend territories (Garrick and Lang, 1977). Furthermore, female alligators show nest fidelity from year to year,

returning to the same nest site at the beginning of each reproductive cycle (Elsey et al., 2008).

We investigated the effects of *in ovo* DDE (1,1-dichloro-2, 2-bis[p-chlorophenyl] ethylene) exposure on aspects of learning and behavior of hatchling American alligators. Specifically, cognition of American alligators was evaluated by comparing the performance of exposed individuals to the performance of control individuals in the spatial learning task. Understanding the effects of organochlorine exposure on crocodilian cognition and behavior is important in a number of ways. Organochlorines, such as DDT, can bioaccumulate and are therefore particularly problematic for top predators. Furthermore, many crocodilians live in regions of the world where DDT continues to be used to combat malaria or in areas where the breakdown products of DDT, DDE and DDD (1,1-Bis(p-chlorophenyl)-2,2-dichloroethane), remain in the ecosystem.

A common method used to assess the ability of an animal to learn is to train an animal to perform a task and then repeatedly change the task that is required of the animal. Such repeated changes allow one to observe how quickly the animal learns a new behavior. A habit-reversal experiment is one such assay as it relies on the same basic methodology (Bitterman, 1965). In a habit-reversal experiment animals are presented with two stimuli that are either visually or spatially distinguishable. One of the two stimuli is assigned to be the “correct” choice and consistently produces a reward when selected by the animal. Visual problems reward the correct stimulus regardless of position. Spatial problems

reward a correct location regardless of what stimuli are present at the location. Throughout the experiment animals are rewarded for choosing the pre-determined “correct” choice. Once the animal reaches a predetermined criterion of correct choices, the discriminanda are reversed and the negative stimulus is now given a positive sign and rewarded while the previously positive stimulus is now given a negative sign and if selected no longer produces a reward. Experimenters collect data on the total number of errors committed during each reversal. Animals of various taxa, including pigeons, some fish species, turtles, chickens and rats show progressive improvement and a decrease in the number of errors committed during each reversal (Gatling, 1951; Bitterman et al., 1958; Reid, 1958; Warren, 1960; Warren et al., 1960; Eskin and Bitterman, 1961; Gonzalez et al., 1964; Stearns and Bitterman, 1965). Very little is known about the ability of crocodilians to participate in these types of tasks.

Previous experiments have explored learning abilities in juvenile American alligators as measured by performance in a series of visual and spatial learning experiments. A modified T-maze was used to determine the ability of the spectacled caiman (*Caiman crocodiles)* to reverse a habit formed in a spatial problem (Williams, 1967). Caimans require fewer trials to learn a new arm of a T-maze after each reversal. The spatial learning ability of *Alligator mississippiensis* compared to that of *Crocodylus acutus* has also been studied *(*Gossette and Hombach, 1969). Both species were asked to discriminate between two stimuli based on location and both species showed progressive improvement. American alligators are capable of performing both visual (Araneo and Farmer, unpubl., Fig

2.2) and spatial discrimination tasks (Gossette and Homback, 1969). Testing these hypotheses adds an ecologically important interdisciplinary dimension. Furthermore, these studies may provide insights into the cause of changes to population dynamics in areas of the world where crocodilians are exposed to organochlorine contaminants. This knowledge may prove important for making fully informed decisions about the risks to ecosystems versus the benefit to humans in the continued use of DDT.

Materials and Methods

Alligator eggs were collected from Central Florida in June of 2010 in collaboration with Dr. Guillette. Eggs were collected from Lake Apopka and the cleaner Lake Woodruff within 2 weeks of oviposition. All eggs were incubated in the Guillette laboratory at the University of Florida, Gainesville. Incubation took place in an environmentally controlled room were the temperature and humidity were monitored daily. Eggs were incubated at 100% humidity and 32ϒC, a temperature that produces both males and females (Milnes et al., 2005). On July 4th, 2010 eggs were transported from The University of Florida, Gainesville to the laboratory facilities at The University of Utah. Temperature was monitored throughout transportation and was never allowed to exceed 33 ϒC or drop below 26ϒC. Once the eggs arrived safely at the University of Utah, they were placed in an incubator where temperature and humidity were monitored daily. At the University of Utah eggs were incubated at a temperature of 32 ϒC and 100% humidity.

The experiment contained three treatment groups: (1) group 1, the treatment group made up of Lake Woodruff animals exposed to a topical application of the contaminate DDE *in ovo;* (2) group 2, containing control animals from Lake Woodruff; and (3) group 3, a group of animals from the contaminated Lake Apopka. On the first day of stage 23, a single topical pesticide treatment was administered to the eggshells of the *in ovo* treatment group (1) of Lake Woodruff animals (methodology of Crain et al., Milnes et al. and Spiteri et al.). A 0.1□g DDE/g egg mass (ChemServ. West Chester, PA) treatment dosage was applied to the *in ovo* group. Such concentrations are shown to affect the differentiation of the gonad (Milnes et al., 2005; Matter et al., 1998; Crain 1997). A stock solution of 1 mg/ml DDE was made up by dissolving 5 mg powered DDE in 50 ml of 95% ethanol. This liquid treatment was then applied to each egg based on egg mass. This procedure ensured that all eggs were exposed to a standardized number of □g of DDE based on egg mass. On the day of hatching, experimental animals were weighed and web tags were attached to the left back foot of each hatchling for identification purposes. Between August 16, 2010 and September 29, 2010, a total of 71 alligators hatched, 21 individuals from embryonic group 1, 19 from embryonic group 2; and 32 from embryonic group 3.

Hatchling alligators were trained to perform a spatial learning task utilizing a modified T-maze in which a free space is built into the T-maze (Figure 4.1). Alligators and Caimans can be trained to perform a spatial learning task reliably when return to a home cage is used as a reward (Davidson, 1966; Northcutt and

Heath, 1971). The modified T-maze was utilized for this learning task in order to prevent the alligators from using vocal and olfactory cues in order to navigate the T-maze. By constructing the T-maze with two arms, each originating from a start box and ending at the free space, no matter which arm the alligator chooses the olfactory and auditory cues will increase as the alligator moved towards the free space. However, these cues increased to the same degree in each arm, meaning that these cues may have encouraged the alligator to swim towards the free space but did not aid the animal at the choice point. The single choice point T-maze consisted of a free space, a start box, a choice point and two arms. The start box led to a single choice point, where an individual had to choose between the left or right arm of the maze. Each arm led to a single entrance to the free space. At the end of each arm a ramp was installed. This ramp ensured that the arms could only be one-way streets. As trials progressed, more and more alligators occupied the free space. It was necessary to design a means of preventing the individuals who had already completed their trials from entering the arms of the T-maze. The ramps accomplished this goal. All areas were filled with water 10.2cm deep. Four submersible heaters and two aquarium pumps maintained the water temperature to ensured reduced variability throughout the maze. The circulation of water by the aquarium pump reduced any chemical cues left behind by the previous alligator. The modified T-maze was maintained at a temperature of 30ϒC (Figure 4.1).

Animals were trained to swim in the T-maze using a progressive protocol that culminated in data collection. The goal of the first step of the training protocol

was to train the alligators to swim down the arms of the T-maze as well as train them to expect both doors to provide entry back into the free space. Animals were trained five days a week, Monday through Friday. Training began by removing the alligators from the free space of the T-maze and placing them in a smaller holding tank. The holding tank contained 10.2cm of water maintained at a temperature of 30°C by a submersible aquarium heater. At the beginning of each trial, a single animal would be removed from the holding container and placed in the start box. During training session both arms were open and led to the free space. A single trial consisted of the experimenter removing a single alligator from the holding tank, placing it in the dry start box and allowing it to walk out off the start box platform and into the T-maze filled with water. At the choice point, the alligator would then have the option of swimming down the left or the right arm of the T-maze. The alligator was considered to have made a choice once all four limbs passed a designated line within the arms of the T-maze, and the experimenter recorded the selection

Initially alligators did not automatically swim away from the experimenter and down the arms of the T-maze when placed in the start box. Most individuals required some encouragement to swim the length of the T-maze arm and walk up the ramp in order to return to the living space. Alligators were motivated by several methods. Experimenter would clap 5.1cm diameter PVC end caps together or shake a piece of plastic tarp over the gators head in order to motivate them to swim. Finally, motivating the alligators to walk up the ramps at the end of the alleyways proved most difficult. In this scenario the experimenter had to

touch the base of the alligators tail with an aquarium net in order to motivate the animal to walk up the ramp initially.

Once all alligators from all groups were swimming the arms of the T-maze without motivation techniques, the second step of the training protocol was begun on the next scheduled day of training. The goal of the second step of the training protocol was to train the alligators to swim through the one-way doors. Each door was constructed from a plastic cafeteria tray and an entry way was drilled in the center of the tray. A piece of black opaque plastic covered the entryway and extended into the water. Velcro was either attached to the top and bottom of the entryway, when the door was “closed”, or simply just to the top when the door was to be “open”. A single one-way door was installed at the end of each arm. By passing through the entryway of these one-way doors the alligator was led onto the ramp and back to the free space of the T-maze. Initially, after the introduction of the one-way door the alligators again need some motivation to swim through the door and walk up the ramp. Once all the individuals were swimming through the one-way doors on a consistent basis the third and final step of the training protocol was begun.

In the third and final step of the training protocol, one by one, an individual would be removed from the holding tank and placed in the start box. The alligator would exit the start box, swim down an arm of the T-maze (thereby choosing between the left and right alleyways), swim through the one-way door, walk up the ramp and be returned to the free space. The alligator’s choice would then be recorded. The one-way doors are not visible to the animal until it enters one of

the arms of the T-maze. During these trials both the left and the right one-way doors were open so that both doors led the animal back to the free space. The goal of the final step of the training protocol was to ensure that there was not a group positional preference. In other words, we wanted to ensure that as a group the alligators did not prefer one arm of the T-maze over another. To test for a group positional preference, all the individual alligators were given a single trial for 4 days in a row. No positional preference was observed. Specifically, the results were as follows in run I 35 alligators selected the left alley way, 37 alligators selected the right alley way, in run II 32 alligators selected the left alley way, 40 animals selected the right alley way, in run III 33 individuals selected the left alley way, 39 alligators selected the right alley way and in run IV 32 animals selected the left alley way, 40 alligators selected the right alley way. After establishing that a group positional preference did not exist, data collection was begun on the next scheduled day of training.

During each data collection trial, one arm of the T-maze, the “incorrect” arm led to a closed one-way door and the "correct" arm lead to an open one-way door. Only by selecting the correct arm will the alligator be able to return to the free space. All individuals were given two to four trials a day; with a 2 hour interval between trials, and the number of incorrect trials were recorded. Each correct trial was rewarded with entrance into the free space. However, if an animal selected the incorrect arm of the T-maze, once it reached the one-way door, the animal was picked up and moved to a small non-home cage container were the animal would be housed individually for the 2 hour interval trial. After

this 2 hour period all the animals were again placed in the holding container and the next scheduled trial was begun. The total number of correct and incorrect trials was recorded for every individual in each embryonic group and the group average was calculated.

The "correct" choice was reversed after all three embryonic groups achieved a group average of at least 70% correct trials in a single day for 4 days in a row. At each reversal the signs of the stimuli were reversed. The mean number of errors was used to measure the performance of each group throughout both Reversal 0 and Reversal 1. Group performance data were then tested for significance with a linear model that compared the slope and intercept of each group. A Chi-squared test was used to compare the performance of each embryonic group in Reversal 0 to their performance in Reversal 1. Alligators and Caimans can be trained to perform a spatial learning task reliably when return to a home cage is used as a reward (Davidson 1966; Northcutt and Heath, 1971). However, in our experiment we observed that alligators were selecting the arm of the T-maze that led to the small non-home cage containers instead of the arm that lead to the free space of the T-maze. We also observed that there was a different rate of learning between the three embryonic groups. We were concerned that this rate of learning was the result of motivation difference between the three groups. To resolve this difficulty we designed a preference test to uncover any motivation differences.

Preference Test

This study consisted of an assay to measure a motivation difference between the three embryonic groups. During each trial, an alligator was placed in a starting location and then was presented with a group of six conspecifics (two individuals from each embryonic group) on one side of an arena while the other side of the arena would be empty. The goal of this study was to determine whether a motivation difference existed between our three embryonic groups. For example, if embryonic group 3 preferred conspecifics to the non-home cage containers while, group 2 preferred the non-home cage containers to conspecifics then any results we potentially saw in our T-maze experiment could be the result of a motivation difference and not an effect of DDE exposure.

For each trial of the preference test, a single alligator was placed in an arena. The arena was 1.83 m long by .61 m wide. The arena was marked at 2 foot intervals from the start position. The arena was maintained at 24^{o}C. The arena was constructed in a quite hallway. Red bricks, to prevent the alligators from escaping, surrounded the perimeter of arena. Because the arena was built in a hallway, we were able to utilize a doorway that led to an office. A Styrofoam box was attached to fishing line and the fishing line was then strung over the office door. This allowed the experimenter to pull the Styrofoam Box from behind the office door. A single animal was placed at the starting location underneath the Styrofoam box, this starting location was equidistant from either end of the dry arena. An experimenter put all the alligators in the holding box. Then a single alligator was randomly pulled from the holding box and placed in a pillowcase.

Placing the animal in a pillowcase ensured that both the animal and the experimenter remained naive to the position of the alligator once the alligator was placed underneath the Styrofoam box. The experimenter then walked behind the door and the trial started. The animal was given 1 minute to orient in box, after one minute the experimenter pulled the box up, exposing the alligator to the arena, using the fishing line. After pulling the box, the animal was given an additional two minutes to make a selection. After two minutes the experimenter opened the office door and the alligator's position in the arena was recorded. In order to measure the position of the alligator, a single piece of tape was placed on the floor of the arena at the location of the body part closest to the left side of the arena. The distance of this piece of tape from the left side of the arena was measured and recorded.

Results

During Reversal 0, embryonic group 1 required 208 trials, embryonic group 2 required 182 trials and embryonic group 3 required 403 trials (Figure 4.2). All the groups required fewer trials to reach criterion in Reversal 1. In Reversal 1 group 1 required 175 trials, group 2 required 147 trials and group 3 required 260 trials (Figure 4.4). Only group 3 showed a significant improvement in the total errors committed in a reversal, between Reversal 0 and Reversal 1 (Chi squared value = 30.84, with 1 degree of freedom, significant at the .01 level). Overall, an initial increase in group error percentage was seen between Reversal 0 and Reversal 1 (Figures 4.3 and 4.4). In both reversals, group 3 had the shallowest slope, whereas group 2 had the steepest slope with group 1 in

between the two (Figure 4.3). Overall, the trend shows an initial mastery of the problem, a dramatic increase in errors percentage during reversals 1 followed by a steady improvement and an overall decrease in total trials (Figure 4.4). Errors may have increased during reversal 1, as animals tend to persist in selecting the stimulous that previously produced a reward but as the animal's experience continues, its selection habit becomes more flexible (Bitterman, 1965).

During Reversal 0, group 2, containing alligators from Lake Woodruff, reached the criterion of 30% average error percentage two calendar days before group 3, the group containing alligators retrieved from Lake Apopka (Reversal 1 was initiated in between block 6 and 7). Group 2 reached these criteria 2 trials before group 1. During Reversal 1, embryonic group 2 reached the criteria of 30% average error percentage one calendar day before embryonic group 1 and 2 trials before group 1. Group 2 reached the criteria of 30% average error percentage 3 calendar days before group 3 and 4 trials before the group 3 (Figure 4.2).

Within the preference test, no difference was seen in the selection made by the individuals from the three treatment groups. Specifically, most individuals chose to remain in the middle of the arena. 77% of group 3, 75% of group 2 and 76% of group 1. Therefore, we can assume any difference seen in the performance of individuals in the T-maze is the result of DDE exposure and not a difference in motivation or reward values.

Discussion

Serial reversal experiments present two problems. One, the animal needs to learn to associate a stimulus with a reward. Two, an animal must learn that its response to the stimulus must be flexible in order to continually receive the reward. DDE exposure appears to affect the acquisition of the first task described above. However, it does not seem to affect the utilization of this task. In other words, DDE appears to affect an alligator's ability to learn the association between a physical response and a stimulus, but DDE exposure does not appear to affect an alligator's ability to utilize the physical task in novel situations.

Individuals from Lake Apopka took longer to learn the association presented in reversal 0, implying that DDE alters the ability to learn and acquirer a new task. However, DDE does not seem to perturb an animal's ability to utilize this task in a novel situation such as that represented in reversal 1. Field and laboratory evidence show that alligator populations are susceptible to the contaminant DDE. Specifically, DDE exposure alters sexual characteristics, hatch rates, egg viability and longevity of this species (Woodward et al., 1993; Crain et al., 1998; Guillette et al., 1999, 2000). However, little is known about the effects DDE has on the behavior and learning ability of these organisms. Understanding the effects on cognition and behavior of organochlorine exposure is important because the ability to create and store new memories and associations regarding one's environment offers a substantial fitness advantage. The current research employed a serial reversal experiment to address the question of whether or not these abilities are perturbed by DDE exposure. A

decreased memory capacity could have a substantial affect on alligator populations because memory is an important element of their life-history strategy. For example, female alligators show nest fidelity from year to year (Elsey et al., 2008), meaning that not only do alligators have the ability to navigate complex environments; they are able to remember locations. This sophisticated behavior may improve hatchling survival and affect sex ratios because in the alligator sex is determined by incubation temperature. By selecting appropriate nest sites from year to year females may increase the survival of hatchlings by remembering good quality nest sites and potentially avoid poor quality nest sites.

Alligators from the contaminated Lake Apopka, committed a greater number of errors while performing in a learning task, however, this group also showed a significant improvement in the number of errors committed during a second reversal. Due to the lack of a differential effect between the topical treatment group and the group from Lake Apopka, the timing of exposure may also be affecting the spatial discrimination ability of this species. If DDE is detrimental to an animal's ability to create new memories this could affect a variety of ecologically important behaviors. In American alligators, exposure to sustained chronic levels of environmental organochlorines also negatively affects embryonic development and is associated with a variety of organizational alterations to the reproductive and endocrine systems of hatchlings and juveniles. Such physiological and behavioral abnormalities have the potential to persist into adulthood and change the population dynamics, genetic diversity,

and potentially even the persistence of this species (Guillette et al., 1995; Milnes et al., 2004).

Through maternal transfer and their action as endocrine disrupting chemicals (EDCs), organochlorines have the potential to modify the hormonal environment by exerting effects on the endocrine system of these animals from the moment of conception (Guillette et al., 1994, 1996b, 1997, 1999, 2000; Crain et al., 1997, 1998; Pickford et al., 2000). Hormonal timing and events are critically important to the development of the brain and the central nervous system. In the current study, a topical treatment was applied during a late stage of development. Development then continues during posthatching. Our late-stage topical treatment combined with posthatching, in a clean environment, may have rescued or permitted a recovery of, our treated group, from any developmental damage. However, exposure through maternal transfer spanned *in ovo* development in our embryos from Lake Apopka. Such exposure has potentially devastating effects given high enough organochlorine concentrations. The presence of EDCs, such as DDT and its metabolites, alter the quantity and timing of hormone production (Crews and McLachlan 2005). Such changes can lead to behavioral abnormalities in animals exposed to EDC contaminants (Gonçalves et al., 2008). Plasma testosterone (T) concentrations decrease in male alligators and plasma estradiol 17-□ (E2) levels increase in females living in contaminated waters (Guillette et al., 1994, 1996b, 1997, 1999, 2000; Crain et al., 1997; Pickford et al., 2000). By modifying the hormonal environment during development, EDCs alter brain pathways and cause multiple neurological

problems (Crews and Mclachlan, 2005). Therefore, changes in the hormonal milieu caused by the presence of EDCs, such as DDT and its metabolites, could account for the multiple changes, including our own results, seen in both field and laboratory alligators exposed to such contaminants. Furthermore, abnormal behavior may indicate that brain development or common decision pathways were perturbed by DDE exposure, such an explanation represents a possible mechanism to explain the differential results we saw in the treated individuals versus the Lake Apopka individuals. Such disruptions have been seen in a variety of other taxa, including humans.

In children, the incidence of learning and locomotor disabilities, autism, impaired hand-eye coordination, memory retention, delayed neurodevelopmental progress and persistent neurobehavioral problems increase with exposure to organochlorines (Keifer and Mahurin, 1997; Colborn 2004; Eskenazi et al., 2006; Iwaniuk et al., 2006). Furthermore, in mice, DDT exposure early in life leads to persistent neurobehavioral problems including impaired performance in maze learning tasks (Eriksson et al., 1990). Neonatal exposure to DDT in mice adversely affects brain development by decreasing the density of muscarinic cholinergic receptors in the cerebral cortex causing a disruption to the overall cholinergic system. Muscrarinic acetylcholine receptors play a role in the encoding of new memories and the use of working memories in humans, rats and monkeys (Tang et al., 1997; Atri et al., 2004; Hasselmo et al., 2004; Green et al., 2005; Winters et al., 2005). A disruption to the cholinergic system, caused by the presence of environmental contaminants such as DDT, has the potential

to adversely affect brain development, resulting in behavioral abnormalities that persist into adulthood (Eriksson et al., 1990). In wildlife, reproductive and behavioral abnormalities and population declines have all been observed in DDT contaminated environments (Fry and Toone, 1981; McLachlan 1993; Fry, 1995; Grasman et al., 1998; Crews and McLachlan, 2005). In the United States, DDT has been banned since 1972. However, due to its slow degradation and the fact that its breakdown products, DDE (1,1-dichloro-2, 2-bis[p-chlorophenyl] ethylene) and DDD (1,1-Bis(p-chlorophenyl)-2,2-dichloroethane), are persistent and ubiquitous, humans and wildlife within the United States are still at risk of exposure (Milnes et al., 2005).

Our first observation, that DDE exposure would affect the spatial discrimination ability of hatchlings was expected. However, we had designed the experiment around the idea that return to the “free space” was a motivating factor for the alligators to perform a physical task. As the experiment progressed a second unexpected observation appeared, animals did not appear to be motivated by return to the free space.

During the experimental trials, if animals selected the incorrect arm of the T-maze, the animal was pulled out of the incorrect arm by the experimenter. The animals were placed in a plastic container, about the size of a shoebox. The animal spent the 2 hour intertrial interval in this box in the experimenter’s office. As the animals’ experience with the experimental procedure increased, a greater percentage of the animals were selecting the incorrect arm of the T-maze. In other words, it did not appear that animals were motivated by return to the free

space. But instead the animals appeared to prefer “the punishment” protocol. All three experimental groups demonstrated this pattern. While the preference test showed that a motivation difference did not exist between the three experimental groups, we are curious to explore what factors may be motivating the alligators to choose the “punishment protocol”. Furthermore, we are curios to see if such choices are based on a recognition, familiarity or habituation to specific experimenters. Future, studies will rely on the protocol established in the current studie’s preference test in order to explore if certain familiar smells, familiar sounds or the presence of a familiar experimenter help to explain this second observation.

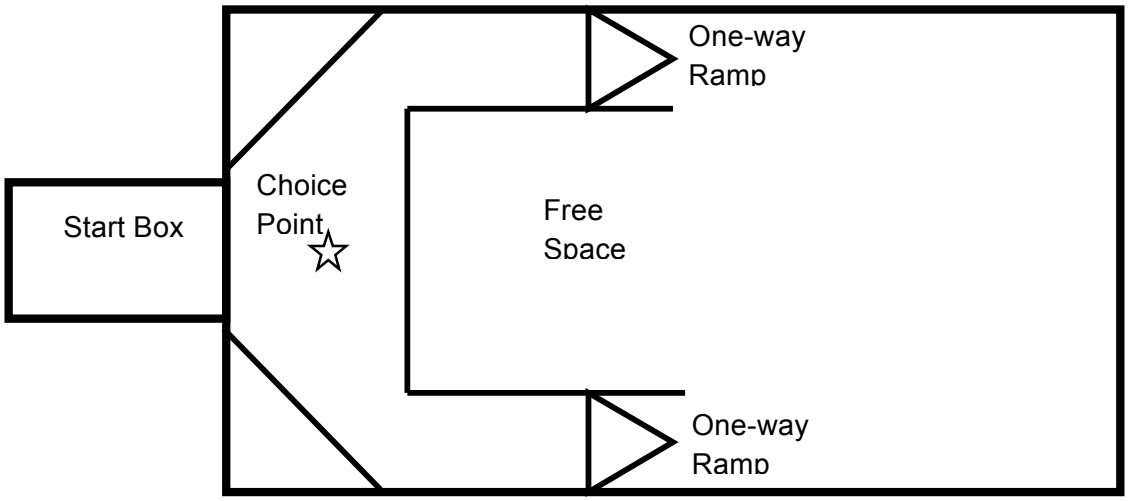

Figure 4.1. Aerial view of T-maze

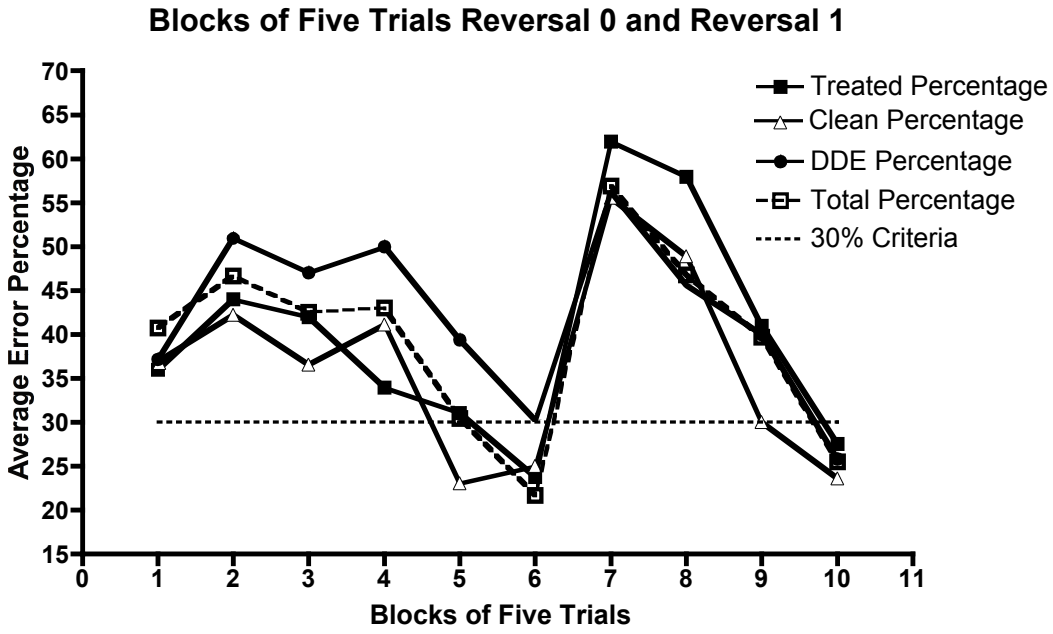

Figure 4.2. Total errors per blocks of five trials in Reversal 0 and Reversal 1

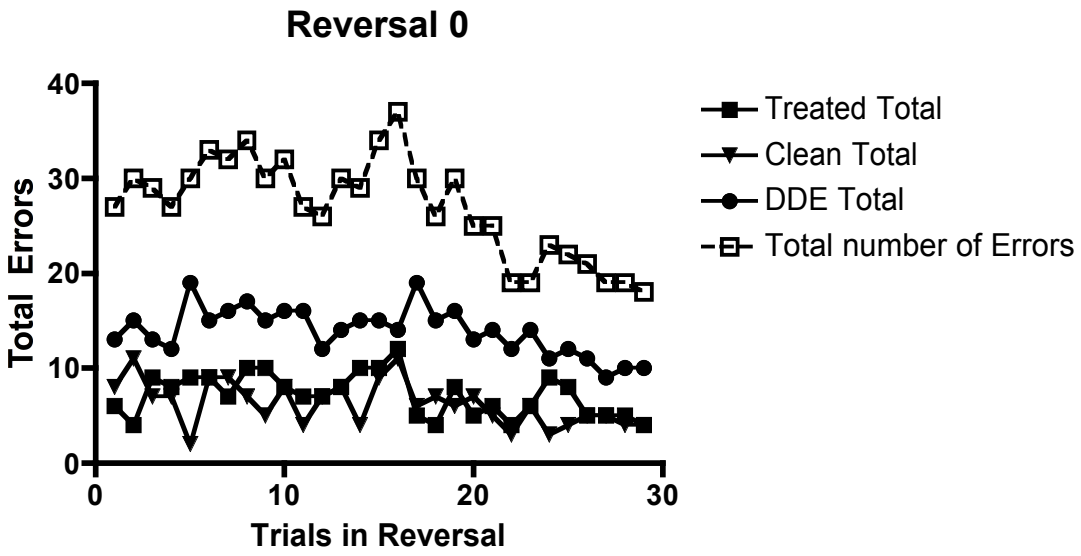

Figure 4.3. Total errors per trial in Reversal 0

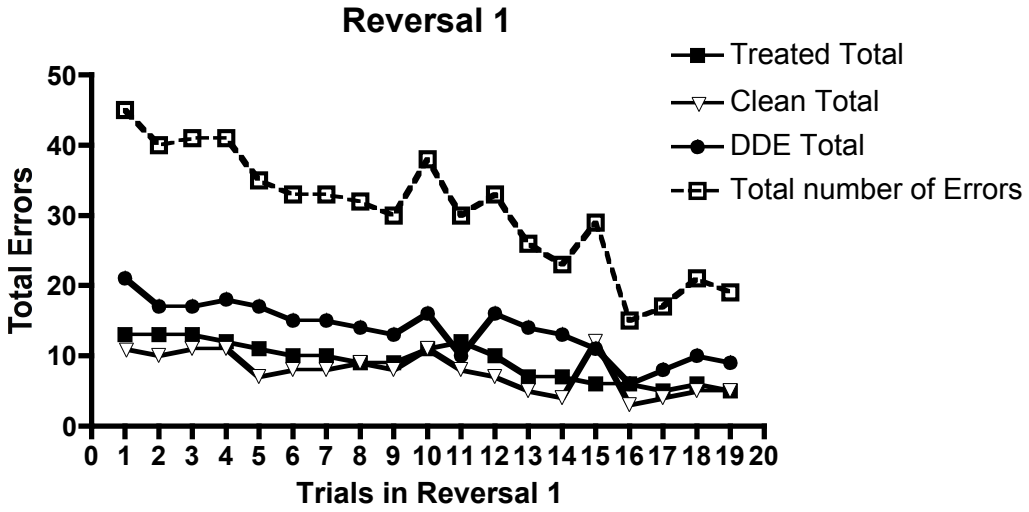

Figure 4.4. Total errors per trial in Reversal 1

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CHAPTER 5

EFFECT OF DDE ON METABOLISM & THERMOREGULATION IN AMERICAN ALLIGATORS

Abstract

We investigated the effect of organochlorines on standard metabolic rate and the preferred body temperature of hatchling American alligators. Standard metabolic rate (SMR) of 27 individuals, from two embryonic groups: (1) embryonic group two, containing control animals from Lake Woodruff; and (2) embryonic group three, animals from the contaminated Lake Apopka, were measured. This investigation is predicated on the concern that contaminants perturb normal brain function and disrupt thermoregulatory behaviors, these behavioral abnormalities, abnormal metabolic rates, and abnormal preferred body temperatures may interfere with population numbers and species survival. SMR was measured by placing an animal in a hermetically sealed respirometer and measuring the amount of O_2 consumed over a period of 6 hours. The current approach, using multiple measurements of metabolic changes, did not reveal significant differences between the two groups, either because there is no difference or that the current approach is not sensitive enough to pick up subtle differences. In light of our observation that a difference in preferred body

temperature was observed in exposed individuals, further investigations may be warranted to reveal subtle differences in metabolic performance.

Introduction

The worldwide use of the synthetic pesticide DDT (1,1,1-trichloro-2,2,-bis p-chlorophenyl ethane) has exposed both humans and wildlife to this pesticide and its breakdown products (Kleinow et al., 1987). A variety of morphological, developmental, and physiological abnormalities in both humans and wildlife have been linked to DDT exposure (Keifer and Mahurin, 1997; Schantz and Widholm, 2001, Colborn, 2004; Iwaniuk et al., 2006; Eskenazi et al., 2006). Furthermore, in wildlife, reproductive and behavioral abnormalities and population declines have all been observed in DDT contaminated environments (Fry and Toone, 1981; McLachlan, 1993; Fry, 1995; Grasman et al., 1998; Crews and McLachlan, 2005).

In the United States, DDT has been banned since 1972. However, due to its slow degradation and the fact that its breakdown products, DDE (1,1-dichloro-2, 2-bis[p-chlorophenyl] ethylene) and DDD (1,1-Bis(p-chlorophenyl)-2,2-dichloroethane), are persistent and ubiquitous, humans and wildlife within the United States are still at risk of exposure (Milnes et al., 2005). DDT continues to be used to combat malaria in sub-Saharan Africa and other warm, humid regions of the world, and thus many wildlife populations continue to be exposed to DDT (Wu et al., 2000). Some of the most biologically diverse and productive ecosystems are tropical humid forests. These ecosystems are home to many endangered species, underscoring the importance of understanding how the

presence of organochlorine compounds, such as DDT, impacts biodiversity in these regions (Wu et al., 2000).

For example, Morelet's crocodile (*Crocodylus morelettie*) is an endangered species inhabiting the Caribbean lowlands of Belize, Guatemala, and Mexico. The chorioallantoic membranes of these crocodiles in Northern Belize contained nine different organochlorine compounds, with DDE being the most common (69%) (Wu et al., 2000). Because Morelet's crocodile is an endangered species, direct research on the effects of these compounds on egg viability, plasma steroid hormone concentrations, and developmental and morphological abnormalities is not an option. However, American alligators (*Alligator mississippiensis)* exposed to organochlorine contaminants, including DDE, show multiple reproductive abnormalities, low clutch viability, reduced phallus size, and altered plasma hormone concentrations (Woodward et al. 1993; Guillette et al., 1994, 1996b, 1997, 1999, 2000; Crain et al., 1998; Pickford et al., 2000).

In American alligators, exposure to sustained chronic levels of environmental organochlorines negatively affects embryonic development and is associated with a variety of organizational alterations to the reproductive and endocrine systems of hatchlings and juveniles. Such abnormalities have the potential to persist into adulthood and change the population dynamics, genetic diversity, and potentially even the behavior of this species (Guillette et al., 1995; Milnes et al., 2004). The eggs and blood of alligators living in contaminated lakes contain elevated concentrations of organochlorines, including DDT and DDE

(Milnes et al., 2006). These elevated levels are associated with decreased egg viability and increased posthatching mortality, which can act to reduce population numbers and threaten the alligators' long-term persistence (Woodward et al., 1993; Milnes et al., 2008).

Reproductive abnormalities have also been linked to organochlorine exposure in alligators. These reproductive abnormalities have the potential to reduce adult fecundity as well as jeopardize genetic diversity. Decreased phallus size is seen in wild male alligators exposed as embryos to organochlorine contaminants, like DDE (Crain et al., 1998; Guillette et al., 1999). Such anatomical abnormalities reduce the male alligator's ability to copulate, leading to diminished adult fecundity and indirectly decreasing genetic diversity. In addition, organochlorines have the ability to alter sex ratios in alligators. Sex in alligators is determined by ambient temperature during a critical period in development. Organochlorine pesticides, like DDT and its metabolites, can override the effects of temperature to produce male to female sex reversals in embryos incubated at temperatures that should produce all males (Crain 1997, 1998; Matter et al., 1998; Rooney 1998).

Embryonic exposure to DDE, in alligators, produces a female bias in clutches incubated at an intermediate temperature capable of producing equal numbers of male and female hatchlings (Milnes et al., 2005). Such altered sex ratios in natural populations could have dramatic effects on genetic diversity by decreasing the sources of paternal genes. In summary, exposure to chronic sustained levels of organochlorines reduces egg viability, decreases hatchling

survival, alters sex ratios, and causes abnormal reproductive tracts in American alligators, and therefore, could have dramatic affects on population dynamics and genetic diversity that could threaten persistence.

The hepatotoxic and other effects of high levels of exposures to organochlorines are relatively well understood but the effects of lower doses, while not immediately lethal, may impact species through effects on metabolic and thermoregulatory abnormalities. Although, the effect of organochlorines on metabolism and preferred body temperature have been explored in arthopod, fish, and bird species few studies have specifically assessed the effects of these molecules on the thermoregulatory system in crocodilians (Ludwig, 1946; Peterson and Anderson, 1969; Jefferies and French, 1970; Leffler, 1970; Verreault et al., 2007).

A variety of chemical agents, including organochlorides such as DDT, are known to affect the thermoregulatory system of a variety of taxa (Gordon, 1994; Gordon et al., 1988). In humans, DDT exposure is linked to a decrease in metabolic rate (Pelletier et al.,2002; Tremblay et al., 2004). DDT exposure in the Japanese beetle (*Popillia japonica* Newman) produced a two-fold increase in the metabolic rate of larvae, while a similar dose quadrupled the metabolic rate of the adult beetle (Ludwig, 1946). Similarly, in blue crabs (*Callinectes sapidus*) a three to five fold increase in oxygen consumption was observed after expose to DDT (Leffler, 1970). Exposure of Atlantic salmon (*Salmo salar*) to DDT produced differential results. When fish were exposed to a high dose of DDT, an increase in SMR was observed, while low dose exposure produced a decrease in SMR in

this species. In gulls high doses of DDT were associated with a decreased Basal Metabolic Rate (BMR). In addition to changes in BMR with exposure of sea gulls to DDT, Jefferies and French (1970) found that when domestic pigeons (*Columba livia domestica*) were exposed to an oral treatment of DDT, not only did the oxygen consumption of these birds change, body temperature and thyroid hormone concentrations changed as well.

In vertebrates, the thyroid hormones, thyroxin (T_4) and triiodothyronine (T_3) directly influence growth and metabolism. Furthermore, these hormones are indirectly involved in development of the central nervous system and skeletal system. Finally, these hormones are involved indirectly in maturation and regulation of the reproductive system (Crain et al., 1998). In addition to domestic pigeons, decreased thyroxin levels have been observed in rats exposed to PCBs and DDT (Bastomsky, 1974; Byrne et al., 1987). In lizards, elevated T_4 concentrations are known to stimulate SMR (Chiu et al., 1970; John-Adler, 1983; Hulbert and Williams, 1988). Furthermore, elevated levels of plasma thyroxin are seen in juvenile male and female alligators living in contaminated lakes (in female alligators only a trend of elevated thyroxin levels was observed $p=0.053$) (Crain et al., 1998). DDT exposure is linked to a variety of morphological, physiological and reproductive abnormalities in American alligators. However, it is not known how elevated thyroxin levels affect the thermoregulatory behavior and metabolism of American alligators. A change in thyroid or other hormone levels, as seen in juvenile male alligators from contaminated lakes, may alter SMR of alligators exposed to such contaminants. It is important to know, if

changes in standard metabolic rates occur with exposure because such a change could affect the health, the food budgets, the energy use patterns and the growth rates of animals. Furthermore, if contaminants are able to perturb normal brain function and disrupt thermoregulatory behaviors, these behavioral abnormalities, may indirectly alter metabolic rates, and interfere with population numbers and long-term species survival.

Materials and Methods

Alligator eggs were collected from Central Florida in June of 2010 in collaboration with Dr. Guillette. Eggs were collected from Lake Apopka and the cleaner Lake Woodruff within 2 weeks of oviposition. All eggs were incubated in the Guillette laboratory at the University of Florida, Gainesville. Incubation took place in an environmentally controlled room were the temperature and humidity were monitored daily. Eggs were incubated at 100% humidity and 32ϒC, a temperature that produces both males and females (Milnes et al., 2005). On July 4th, 2010 eggs were transported from The University of Florida, Gainesville to the laboratory facilities at The University of Utah. Temperature was monitored throughout transportation and was never allowed to exceed 33 ϒC or drop below 26ϒC. Once the eggs arrived at the University of Utah, they were placed in an incubator where temperature and humidity were monitored daily. At the University of Utah eggs were incubated at a temperature of 32 ϒC and 100% humidity.

The experiment contained two embryonic groups: (1) group two, containing control animals from Lake Woodruff; and (2) group three, a group of animals from the contaminated Lake Apopka. On the day of hatching, experimental animals were weighed and web tags were attached to the left back foot of each hatchling for identification purposes. Between August 16, 2010 and September 29, 2010, 19 from group 1; and 32 from group 2.

Twenty-seven captive raised American alligators were housed in an approved animal care facility on the University of Utah campus. These 27 individuals were divided between two separate plastic 25-gallon containers. Because the animals were living in a green house facility, they experienced natural light and photoperiods. Each 25-gallon container contained 10.2 cm of water maintained at 30°C by a submersible aquarium heater. Animals were maintained on a diet of Mazuri Brand commercial gator chow. The animals ranged in mass from 47.32 to 38.47 g, with a mean mass of 41.3 g. The volume of these animals ranged from 54.5 to 39.1 ml, with a mean volume of 45.7 ml.

Standard metabolic rate was measured by placing an animal in a hermetically sealed respirometer to measure the amount of O_2 consumed over a period of 6 hours (as described in Farmer and Carrier, 2000a, 200b). The respirometer was constructed from a PVC pipe (35.6 cm long with an inside diameter of 5.1 cm and a total volume of 316 ml) and PVC end caps. Epoxy was used to seal the end caps to the PVC pipe. The respirometer also contained 42 ml of Soda Sorb. During each round of data collection nine alligators were brought down from the animal care facility and placed in an environmentally

controlled experimental room overnight. All animals had been fasted for at least 48 hours. Animals were placed in the PVC pipes, the pipes were sealed and then filled with 100% humidified air with a known percentage of O_2 and CO_2. After six hours, the oxygen consumption (V_{O2}) and carbon dioxide production (CO_2) were obtained for each animal by sampling a fraction of the volume of air from each respirometer using oxygen (Ametek S-3A, Pittsburgh, PA, USA) and carbon dioxide (Ametek CD-3A, Pittsburgh, PA, USA) analyzers. Each respirometer was also equipped with a thermistor. Throughout the 6 hour period, heat production was also measured utilizing thermistors that continuously monitored the air temperature of a single respirometer.

Each thermistor had been previously calibrated using a high temperature standard of 50.4°C and a low temperature standard of 19.4°C. Temperature, O_2 and CO_2 signals were all sampled at a rate of 50 Hz. Each gas analyzer and thermistor was monitored independently by a single channel of an AD converter (Biopac System, Goleta, CA). These digital signals were visualized on a Macintosh computer and analyzed using Acknowledge software (Biopac System, Goleta, CA) data were then saved on the same computer.

O_2 consumption, CO_2 production and heat production P values were calculated utilizing a linear regression in order to shine light on the relationship between treatment and any of our measured values. To remove the effect of mass on SMR measurements, we transformed the VO_2 and body mass data into a linear model using a *log* function. Then to compare *log*VO_2 and *log* body mass we found the residuals of the linear function for both DDE exposed individuals

and clean individuals. These group residuals were then plotted against body mass using a Residual dependence plot that plots the residuals against the original horizontal axis, and assigns a best fit line to the values. This allows us to see the variation in residuals. Finally, we then calculated the mean, standard deviation and standard error of the residuals for each treatment group.

In a second experiment a thermogradient was used to determine the effect of contaminant exposure on preferred body temperature by comparing the thermoregulatory behavior of a group of DDE exposed animals to a group of control animals. Three rows of infrared bulbs were used to establish the temperature gradient. The first row was contained two 75 watt bulbs placed 80 cm above the base of the tank. The second row contained two 100 watt bulbs placed 117 cm above the base of the tank. The third row was positioned at the very back edge of the tank and contained two 100 watt bulbs at a height of 108 cm above the tank. The rows of heat lamps created a temperature gradient ranging from 21.5°C to 35°C. Each alligator was placed in the middle at the front of the tank where the temperature was 21.5°C. The animals were instrumented with a temperature data logger (I-Button, Maxim Integrated Products). Data loggers were secured to the backs of the alligators using electrical tape. The data loggers recorded temperature once every 2 minutes for 8 hours. The mode for each individual was calculated. These modes were then tallied to create a histogram. A chi-squared test was utilized to test for significance between the preferred body temperature of control and DDE exposed individuals. Additionally,

we used an ANOVA and linear regression to look at the effect of mass on preferred body temperature.

Results

Overall no effect of treatment was seen on O_2 consumption, CO_2 production or heat production between treatments groups. Specifically, the percentage of total O_2 consumed was compared between DDE exposed individuals and alligators from the cleaner Lake Woodruff ($p=0.392$). If one group consumed a greater percentage of the total O_2 available, the points of the graph would be shifted to one side of the diagonal. However, all points align along the diagonal, meaning a treatment effect was not seen between the two embryonic groups (Figure 5.1).

Similarly, no separation was observed between the groups in O_2 Residuals. This residual plot compared the differences between data points (i.e., residuals) of DDE exposed individuals and alligators from the cleaner Lake Woodruff versus all (pooled) residuals, for the percentage of total O_2 consumed. The residual plot shows that the data distribution was the same for both DDE exposed individuals and alligators from the cleaner Lake Woodruff (Figure 5.2). A quantile quantile analysis was also run for the difference in CO_2 production between the two treatment groups (Figure 5.3).

No difference was seen in CO_2 production between embryonic groups ($p= 0.0679$) (Figure 5.3). A similar result was produced in a CO_2 production Residuals analysis, by comparing residuals of DDE exposed individuals and

alligators from the cleaner Lake Woodruff versus pooled residuals for CO_2 production (Figure 5.4). Finally heat production was analyzed in two ways. One, a quantile quantile plot was utilized to compare the difference in maximum respirometer temperature and minimum respirometer temperature (max - min) between DDE exposed individuals and alligators from the cleaner Lake Woodruff (Figure 5.5). No difference in heat production was observed between individuals exposed to DDE and individuals from Lake Woodruff ($p=0.873$). Two, a residuals analysis was run to compare the heat production residuals of embryonic Group A and Group B versus pooled residuals for differences between room temp (max-min). Finally, no difference was observed in heat production between DDE exposed individuals and alligators from the cleaner Lake Woodruff (Figure 5.5). The results of our linear transformation are presented in Figure 5.6 and Figure 5.7. Furthermore, the individual group residuals are presented in Figure 5.8 and 5.9. The mean of the residuals of the mass independent O_2 consumption of exposed individuals is -1.9×10^{-17}. The standard deviation for this group's residuals is 0.280 and the standard error is 0.075.

While a treatment effect was not observed for metabolic measurements. A difference in preferred body temperature was observed between embryonic groups. The mode of the 30 hatchlings was calculated and tallied to create a histogram (Figure 5.10). For example, 6 DDE exposed individuals had a mode at 32 °C, while 4 control individuals had a mode of 24.5°C.The control individuals represent a larger spread across the temperature gradient. The DDE exposed individuals represent a spread surrounding 32°C. A clear trend of separation

between the preferred body temperature of the control animals and DDE exposed animals is observed. A chi-squared test was used to compare the frequencies of the two treatments. A p value of less than .001 was found; meaning DDE exposed individuals had a different preferred body temperature. Additionally, we also considered the effect of alligator mass on preferred body temperature. While our heaviest individuals were from our DDE exposed group, overall the weights of the two treatment groups were comparable (Figure 5.11). After running an ANOVA, we saw that mass did not have a significant effect on the preferred body temperature of the control group (p=0.3419). However, mass was an independent factor on preferred body temperature for DDE exposed individuals (p=.015).

Discussion

Our data suggest no difference between O_2 consumption or CO_2 production between individuals exposed to DDE and unexposed individuals. Furthermore, when we look at the residuals from all the individuals in a single treatment group, we observe intra group variation in these residual values. However, when we compare the residuals from one treatment group to the residuals of the other treatment group, we see that this intra group variation is similar for both groups and therefore we conclude that treatment did not affect SMR.

Braham and Neal (1974) measured metabolic rate of shrews and found that after exposure to DDE, individuals showed an increased metabolic rate. However, this effect was transient and within 6 days after exposure it was

observed that the metabolic rate of exposed animals had returned to the previous level. Braham and Neal (1974) concluded that this transient effect was due to clearance of the organochlorine from the animal's body.

In the present study, there is precedence for maternal transfer of organochlorines in a known contaminated environment. In juveniles from this same environment, altered thyroxin levels are observed. Most likely these individuals are exposed to DDE *in ovo* with continued exposure after hatching. Organocholorides act in a similar manner with thyroxin as they do with sex steroids, meaning they competitively bind to thyroxin and reduce the T_4/T_3 ratio, possibly altering measurements of metabolic rate. In our study, animals were exposed through maternal transfer only, and metabolic measurements were taken six months after hatching; leading us to question, like in the shrew, if there was clearance of the organochlorine after hatchling and possibly a transient impact on metabolism. Future studies may address this possibility by tracking metabolic measurements over a time course starting at day of hatching to six months after hatching. If a notable effect is observed, hormone studies could be carried out to compliment these metabolic measurements.

An exceptionally low standard metabolic rate is a cornerstone of crocodilian biology and an adaptation of this clade to its ecological niche. A change in metabolism caused by exposure, either directly through intrinsic physiological responses, or indirectly through modification of preferred body temperature, could modify daily energy expenditures with significant consequences on food requirements, growth rates, and population dynamics.

Understanding the effects of organochlorine exposure on crocodilian metabolism and thermoregulation is important in a number of ways. One, testing these hypotheses adds an ecologically important interdisciplinary dimension. Two, these studies provide insights into population dynamics in areas of the world where crocodilians are still exposed to organochlorine contaminants.

Organochlorines, such as DDT (1,1,1-trichloro-2,2,-bis p-chlorophenyl ethane), can bioaccumulate and are therefore particularly problematic for top predators. Furthermore, many crocodilians live in regions of the world where DDT continues to be used to combat malaria or in areas where the breakdown products of DDT, DDE (1,1-dichloro-2, 2-bis[p-chlorophenyl] ethylene) and DDD (1,1-Bis(p-chlorophenyl)-2,2-dichloroethane), remain in the ecosystem. DDT has been show to affect metabolism in a variety of species, and therefore its continued use may well affect the dynamics of exposed populations (Ludwig 1946; Peterson and Anderson 1969; Leffler 1970; Jefferies and French 1970; Verreault et al. 2007). Crocodilians are a group of animals that are still environmentally exposed, worldwide, to DDT and its breakdown products. However, little is known about the effects this exposure has on the metabolism and thermoregulatory behavior of this species. The current study suggests that maternal transfer may not be adequate to perturb normal metabolic rates in hatchlings. However, in wild populations, animals continue to be exposed to contaminants after hatchling through environmental contact. This continued exposure after hatching has been shown to affect thyroxin levels in these individuals and therefore we hypothesised that altered metabolic rates may only

be observed when individuals are continuously exposed to DDE. Therefore, future metabolic studies should focus on measuring the physiological parameters in animals that have been raised in environments were DDE exposure mimics natural exposure pathways. While *in ovo* DDE exposure did not directly effect metabolism. As ectotherms, metabolism can be indirectly influenced by preferred body temperature and thermoregulatory behavior. Therefore, because DDE affects behavior in alligators and other taxa, it was important to look at the behavioral parameters that may be indirectly controlling metabolic rate as well (Araneo and Farmer, unpubl.; Crews and Mclachlan, 2005).

Even though we did not observe a direct effect of *in ovo* DDE exposure on the metabolic parameters (O_2 consumption and CO_2 utilization) we measured, we felt it was reasonable to measure preferred body temperature because thermoregulatory behavior is a combination of behavior and metabolism. Furthermore, through its action as an endocrine disrupting chemical and DDE's ability to perturb thermoregulatory behavior in other ectothermic species we felt it was possible that exposure could lead to altered brain and behavior patterns. Specifically, early exposure to DDE could alter brain pathways that are responsible for thermoregulatory behavior or the neural components an animal uses to process temperature information. Such altered pathways could persist into adulthood and could continue to cause problems even after the toxin has been cleared from the animal's body and any direct effects on metabolism are no longer observable. However, these altered behavioral patterns could continue to

exert indirect effects on an animal's metabolism, health and reproductive success.

DDE exposure was found to be correlated with a higher preferred body temperature in hatchling American alligators. Specifically, DDE exposed individuals had a preferred body temperature surrounding 32°C, whereas unexposed individuals had a preferred body temperature closer to 24°C. Previous experiments have found that satiated postparandial alligators have a preferred body temperature close to 30°C (Farmer et al., 2008). After consuming a meal alligators will look for a basking location. This behavior makes sense because the elevated temperature of a basking location will facilitate accelerated digestion. However, in order to conserve energy when alligators are fasting, animals usually look for lower temperatures (Farmer, 2008). Given our results, unexposed animals follow the same pattern Farmer observed in laboratory animals. However, our DDE exposed animals do not follow the pattern observed by Farmer. Specifically, we did not expect 48 hour postparandial animals to have a preferred body temperature of 32°C.

Both field and laboratory evidence show that alligator populations are susceptible to the contaminant DDE. Specifically, DDE exposure alters sexual characteristics, hatch rates, egg viability and longevity of this species (Woodward et al., 1993; Crain et al., 1998; Guillette et al., 1999, 2000). However, little is known about the effects DDE has on the thermoregulatory behavior of these organisms. DDE exposure in salmon has been shown to change their preferred water temperature and thus it is possible DDE exposure alters temperature

preferences in other species as well (Ogilirie and Miller, 1979). The current research was also devoted to exploring the effects of DDE (1,1-dichloro-2, 2-bis[p-chlorophenyl] ethylene) exposure *in ovo* to thermal preferences. Numerous aspects of poikilotherm metabolism are affected by environmental temperature (Rome, 1990; Logue et al., 2000; Somero, 2004; Guschina and Harwood, 2006; Bicego et al., 2007). Therefore, it is important to know if preferred body temperatures are perturbed by DDE exposure because these changes could affect the population numbers, the overall health of animals, their reproductive success, and the health and growth rates of hatchlings.

The altered preferred body temperature we observed in animals exposed to DDE could have direct effects on the energy reserves, food budgets and population dynamics of these animals. If animals are not seeking out cooler temperatures when fasting they are going to be unable to conserve energy in the same way as their unexposed counterparts are able. Such inabilities to conserve energy could be particularly problematic during periods when food is not readily available. Furthermore, high temperatures are usually found only on land or in shallow water. Both of these locations make animals more visible to predators increasing the likelihood that animals will become prey. Both of these consequences of an altered preferred body temperature could increase hatchling mortality and reduce the number of individuals that reach adulthood, changing population dynamics and potentially threatening long-term species persistence. It is also possible that DDE exposure may not be directly effecting the preferred body temperature of exposed animals but may instead be changing the ability of

an animal to appropriately sense temperatures or process environmental temperature information. If these changes persist into adulthood they could affect population dynamics.

For example, females that are unable to sense appropriate temperatures may indirectly suffer from altered metabolic rates leading to an inability to concentrate nutrients from their body into the yolk of embryos. Furthermore, female alligators may increase survival of hatchlings by selecting nest sites with appropriate thermal regimes. Additionally, mothers may not only be selecting nest sites with appropriate thermal regimes they may also poses behavioral patterns that help to minimize potential hazards, such as over-heating or drying out. Specifically, American alligator mothers are observed resting their throats on the surface of their nests. This behavior may allow females to use the more sensitive skin on the throat to monitor the temperature and dampness of their nest (Neill, 1971). Female *Crocodylus fasciatus* have been observed lying on top of their nest on sunny days, potentially acting to shad the nest from the sun (Deraniyagal, 1939). American alligators show nest fidelity from year to year (Elsey et al., 2008), meaning by remembering good quality nest sites and potentially avoiding poor quality nest sites, this sophisticated behavior may also improve hatchling survival. Finally, alligator populations are not simply facing contaminant exposure, but like all species they are facing climate change. If contaminants are able to perturb normal brain function and disrupt thermoregulatory behaviors, these animals may be more sensitive to climate change related to the current global climate trends. Behavioral abnormalities,

abnormal metabolic rates, and abnormal preferred body temperatures may interfere with population numbers and species survival. The mechanism for how organochlorides exert their disruptive effects is still under debate. However, to date the most common theory surrounds their action as Endocrine Disrupting Chemicals (EDC).

Hormonal timing and events are critically important to the development of the brain and the central nervous system. The presence of EDCs, such as DDT and its metabolites, alter the quantity and timing of hormone production (Crews and McLachlan, 2005). Therefore, changes in the hormonal milieu caused by the presence of EDCs, such as DDT and its metabolites, could account for the changes seen in animals exposed to such contaminants. By modifying the hormonal environment during development, EDCs alter brain pathways and cause multiple behavioral problems (Crews and Mclachlan, 2005). Such changes to brain pathways could account for the difference in preferred body temperature we observed in hatchlings exposed to DDE compared to the preferred body temperature of unexposed animals. Seeking out cooler temperatures, when fasting, is potentially an important behavioral pattern that allows animals to conserve energy if food is scares. Alterations to this behavioral pattern due to the EDC action of DDE could result in changes in the energy budgets and overall health of exposed individuals.

Energy conservation is an important component of an animal's energy budget. However, energy acquisition, through efficient digestion and absorption, is also an important element that contributes to energy budgets. The current

study addressed how DDE exposure affects energy conservation; future studies could address energy acquisition. After feeding, alligators have been observed seeking out temperatures around 30°C (Farmer et al., 2008). As ectotherms, many aspects of their physiology are influenced by environmental temperatures including the action of digestive enzymes and enzymes involved in nutrient absorption. By seeking out higher temperatures after feeding alligators may be increasing the speed of digestion and increase the efficiency of absorption. Both of which contribute to the animals overall energy acquisition ability. Alterations to the behavioral pattern observed after feeding could therefore affect an animal's energy acquisition ability. Future preferred body temperatures studies could address this aspect of an animal's energy budgets. Such studies would utilize the protocol established in this study. However, instead of measuring the preferred body temperature of fasting animals, we would measure the preferred body temperature of animals for the 48 hours immediately after feeding. If we again see an altered behavioral pattern we could conclude that DDE exposure indirectly effects energy conservation but also energy acquisition through changes to behavioral patterns that contribute to these components of an animal's overall energy budget.

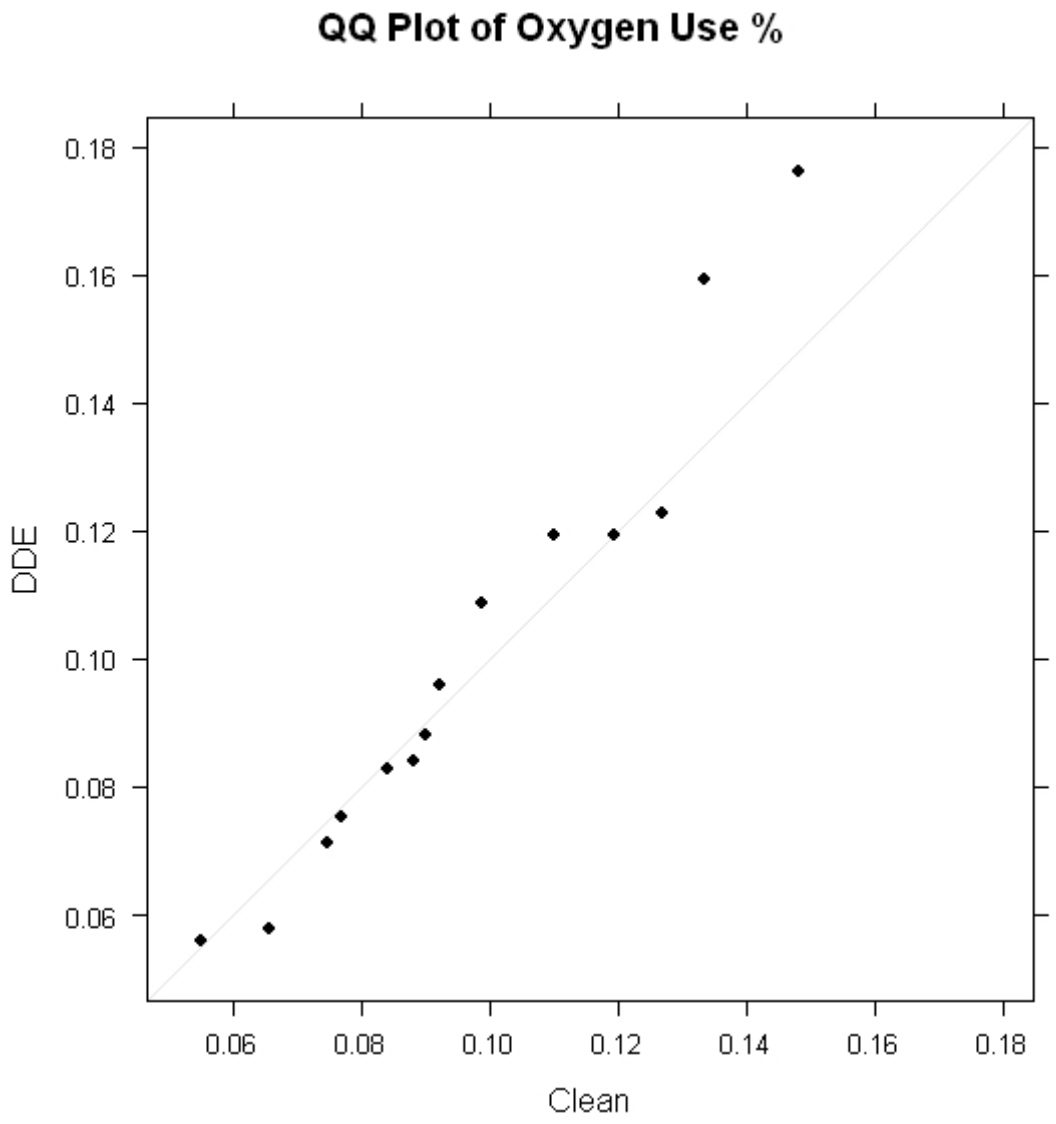


Figure 5.1. Quantile quantile plot of $\%O_2$ use for animals naturally exposed to DDE versus control animals from a clean environment

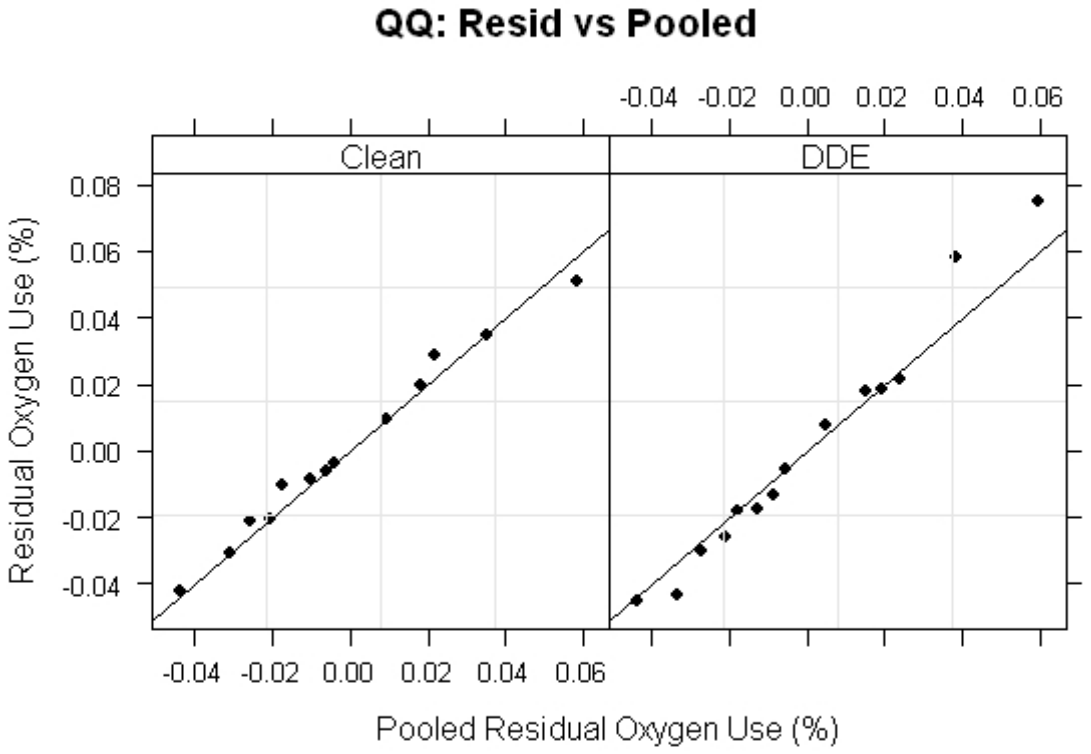


Figure 5.2. Pooled residual plot of $\%O_2$ use for animals naturally exposed to DDE versus control animals from a clean environment

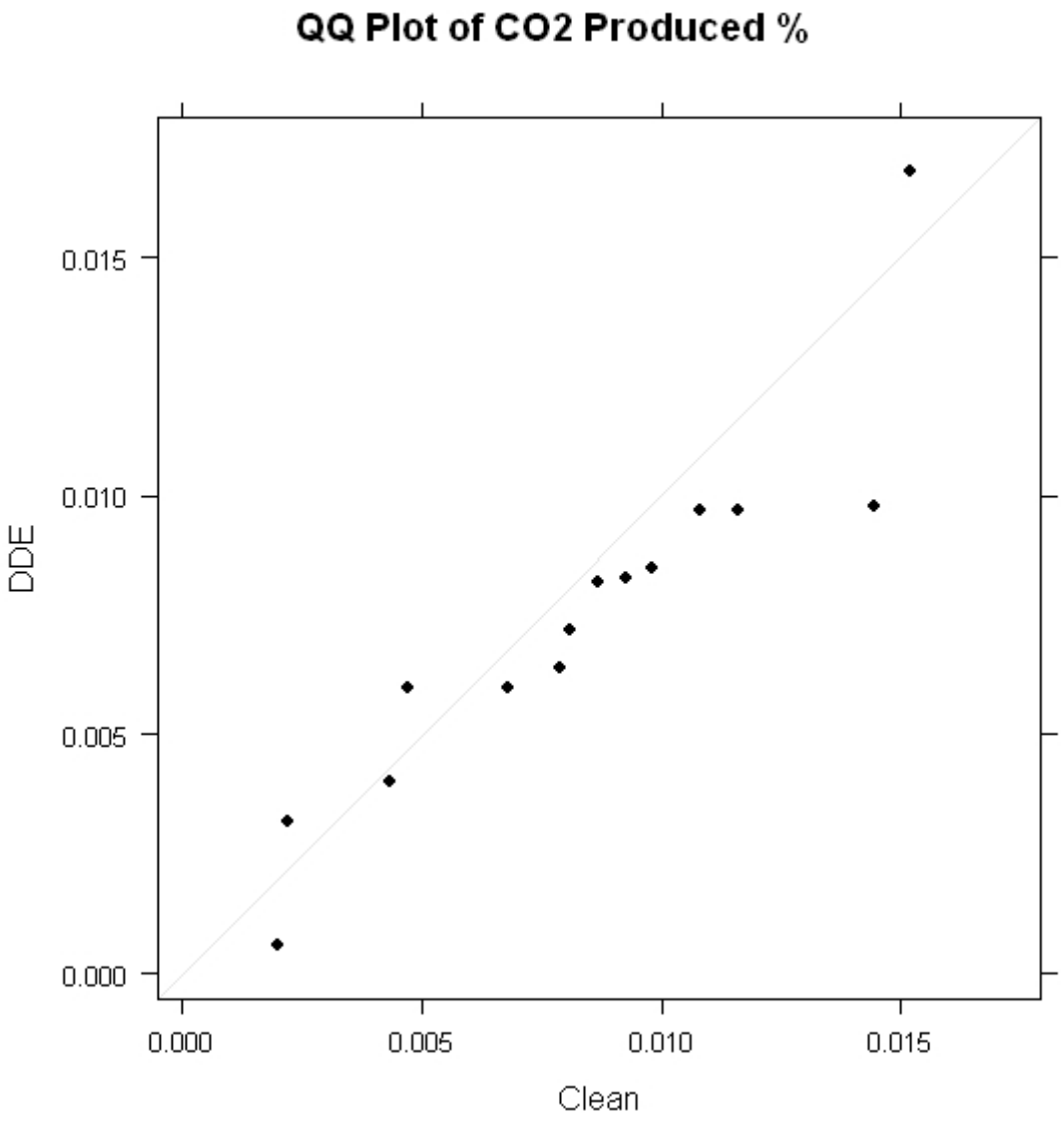


Figure 5.3. Quantile quantile plot of % CO_2 use for animals naturally exposed to DDE versus control animals from a clean environment

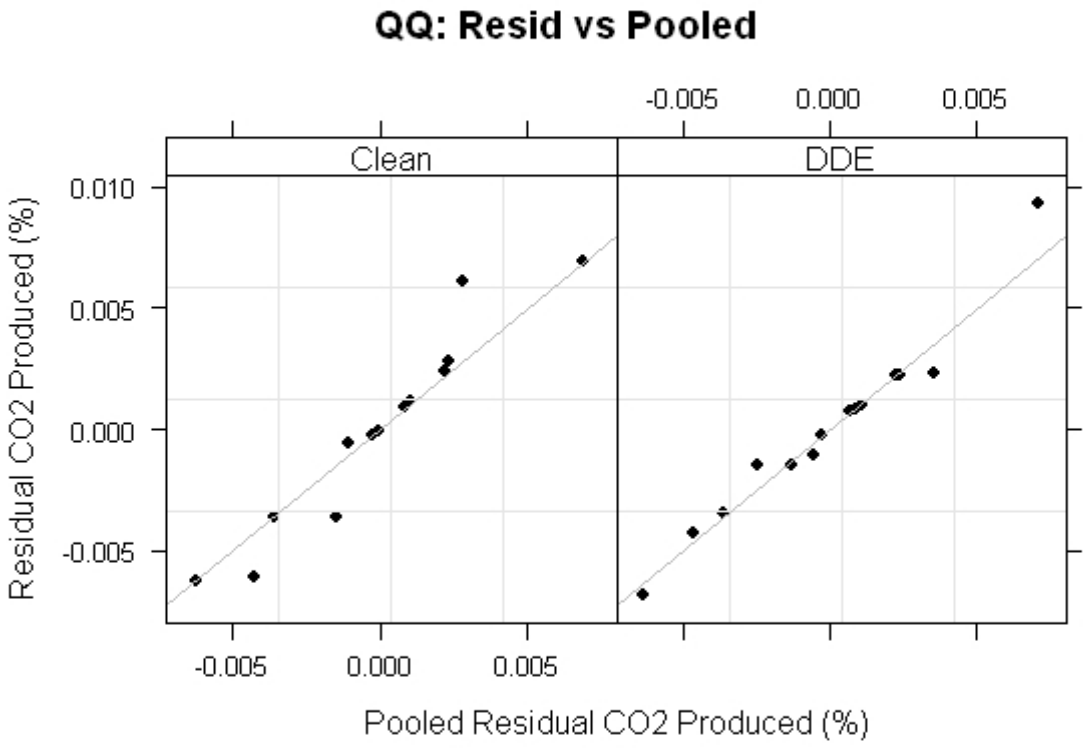


Figure 5.4. Polled residual plot of % CO_2 use for animals naturally exposed to DDE versus control animals from a clean environment

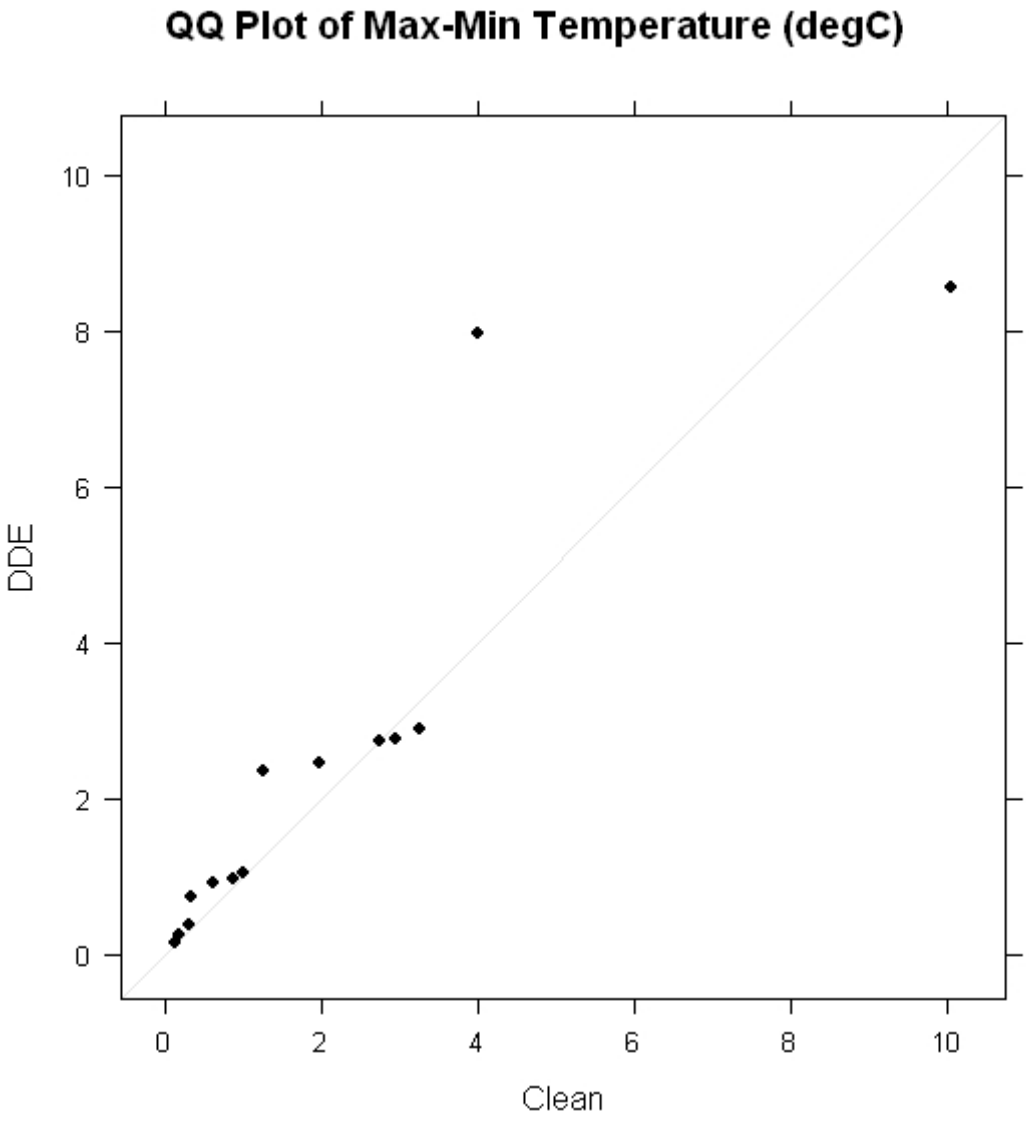


Figure 5.5. Quantile quantile plot of heat production (°C) for animals naturally exposed to DDE versus control animals from a clean environment

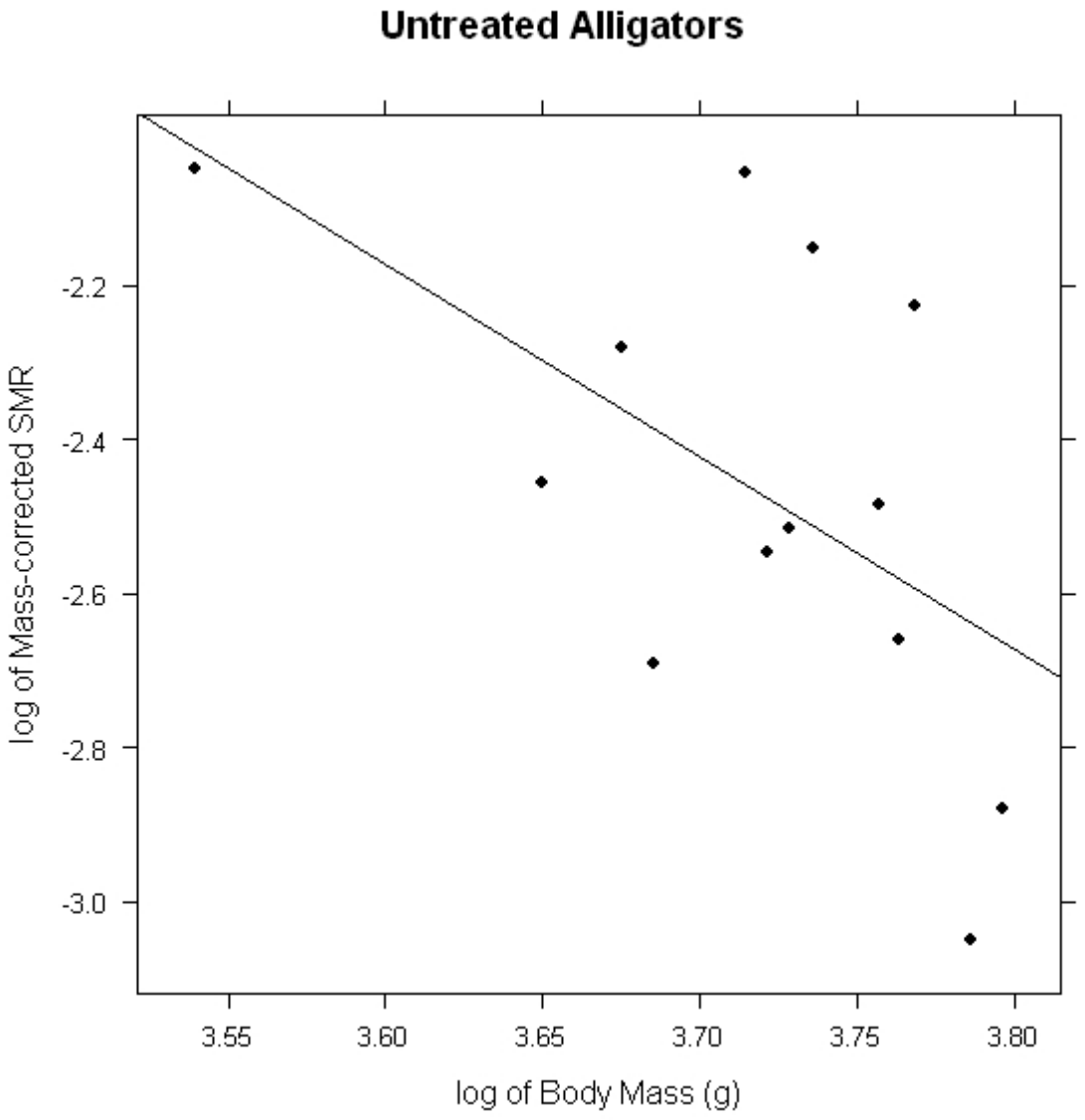


Figure 5.6. Mass corrected logarithmic plot for SMR (ml 0_2/min) for control animals from a clean environment

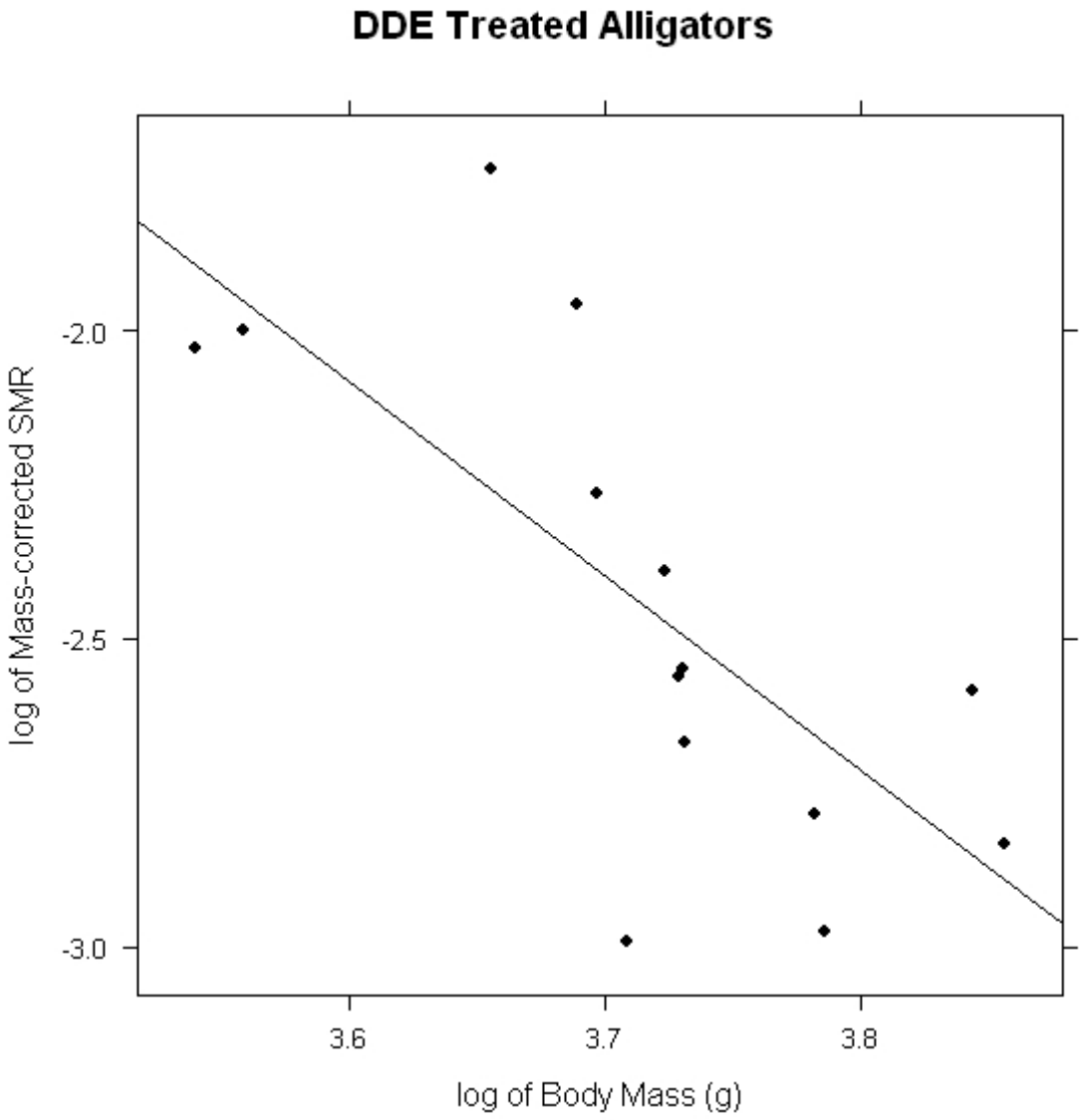
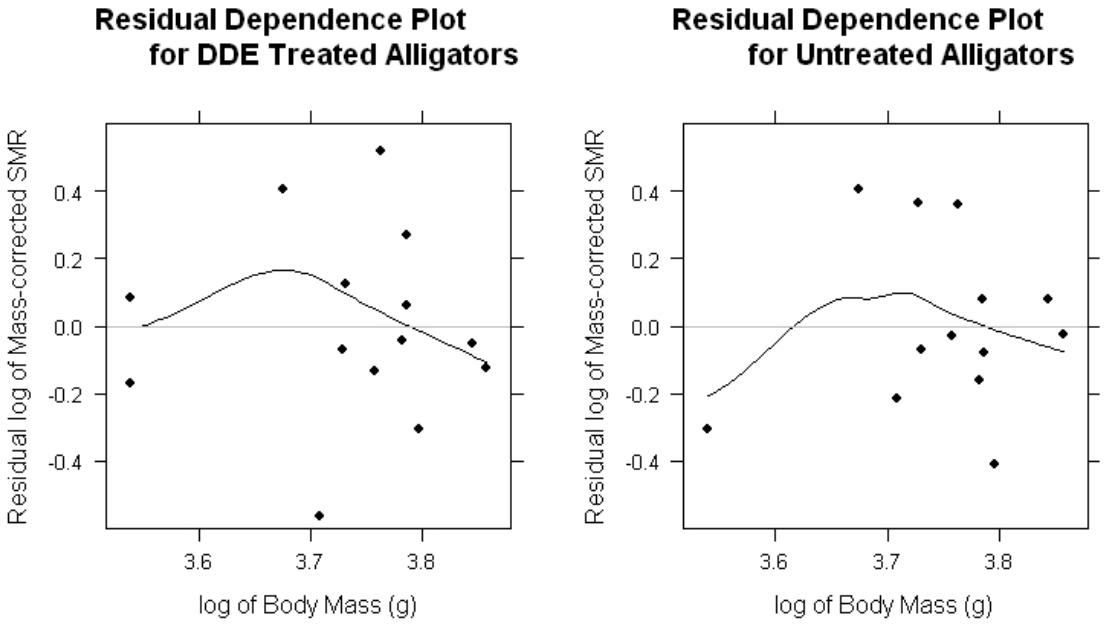


Figure 5.7. Mass corrected logarithmic plot for SMR (ml 0_2/min) for animals naturally exposed to DDE



Figures 5.8. and 5.9. Residual values for mass corrected logarithmic plot for SMR (ml 0_2/min) for control animals from a clean environment and animals naturally exposed to DDE

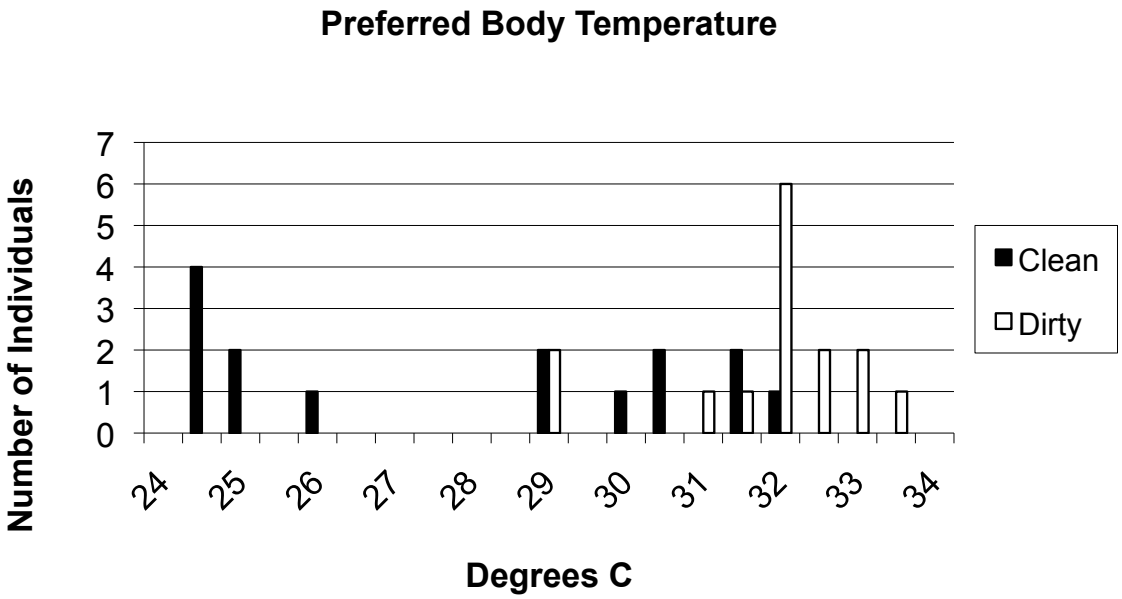


Figure 5.10. Histogram of the preferred body temperature (°C) modes for control animals from a clean environment and animals naturally exposed to DDE

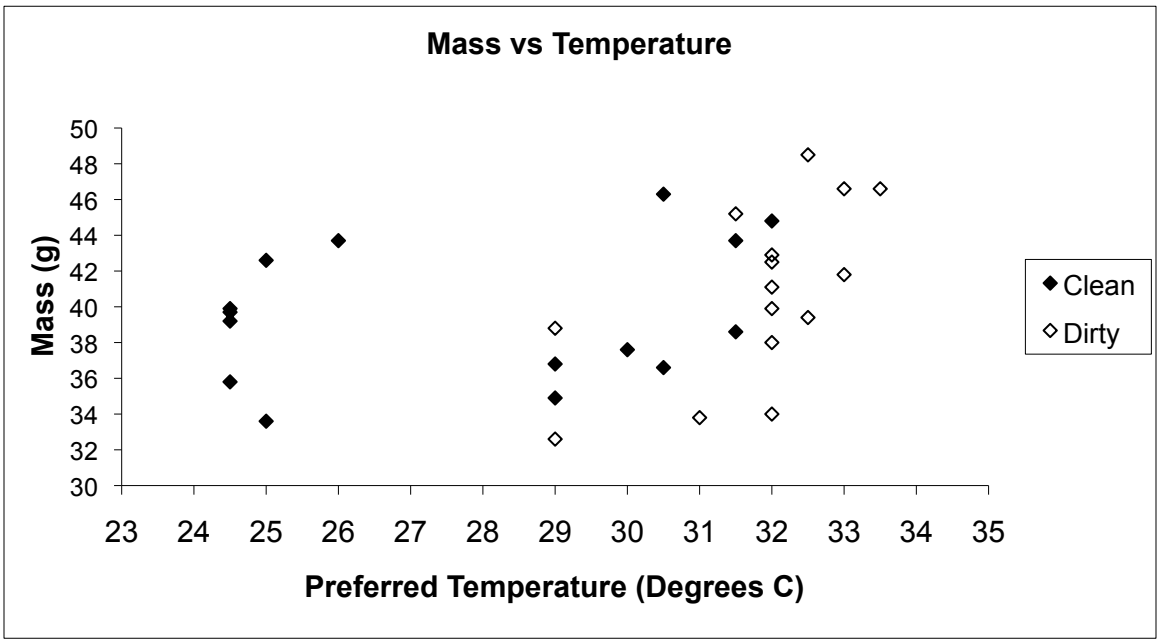


Figure 5.11. Relationship between mass (g) on preferred body temperature (ϒC) of control animals from a clean environment and animals naturally exposed to DDE

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CHAPTER 6

CONCLUSIONS

This research addressed learning in captive, juvenile American alligators using methods that have been shown in other species to be informative. In each one of the previously discussed discrimination studies animals were trained to perform a physical task when presented with a specific set of stimuli. The sign values of the stimuli were then changed repeatedly. In all three discrimination experiments, alligators demonstrated that their cognitive abilities are flexible and that acquired memory can be applied to novel situations. This flexible component may mean that in natural populations, memory utilization and acquisition could be modified by social interactions, environmental interactions and imitation of older members of the species.

Our investigations show definitively that juvenile alligators develop cognitive skills at an early age. Juvenile alligators are capable of performing both visual and spatial discrimination tasks. Furthermore, the visual and spatial discrimination ability of this species may be innate. In other words, because our juvenile laboratory reared animals were able to perform in these discrimination tasks, the discrimination ability of juvenile alligators does not require imitation, imprinting from adults or parental rearing in order to be effectively used by the animal while acquiring a novel task. Additionally, spatial tasks may be simpler, as

they reflect natural behaviors such as navigation and place memory. Furthermore, performance abilities may be contextual, in that performance appears to be affected by temperature and exposure to environmental contaminants.

Chapter 2 established the visual discrimination and learning ability of this species. Additionally, the research presented in chapter two established the optimal protocol that would allow juvenile American alligators to effectively learn a physical task when presented with a food reward and ascertained that in a serial reversal experiment juvenile American alligators show a pattern of decreasing errors as their experience with the problem increased. Reversal experiments such as these present two problems. Each problem offers unique insights into learning processes and memory formation. The first problem allows an animal to demonstrate an ability to associate a physical task with a reward. However, with continual reversal of the positive and negative discriminanda, a second problem arises where the animal is allowed to demonstrate an ability to learn and remember that the responses acquired in the first problem must be flexible. Alligators show a pattern of decreasing errors in a series of successive visual discrimination problems, the pattern observed in alligators is similar to the pattern seen in a variety of bird taxa. Very few studies have investigated the ability of American alligators to participate in these types of tasks. We believe the shared pattern of behavioral flexibility detailed in chapter two supports the prediction that alligators have demonstrable learning capabilities and that these

abilities are innate and develop early in the young even in the absence of parental nurturing, or environmental cues.

The research presented in Chapter 3 documented spatial learning abilities and the effect of temperature on the spatial learning ability of juvenile American alligators. This assessment of spatial learning incorporated two separate temperature treatments - one at the lower end and another at the upper end of the American alligators preferred activity range. A clear trend of decreasing errors with each reversal was observed. In addition, alligators made fewer total errors at 22ϒC than at 32ϒC regardless of what temperature régime the individual experienced first. We conclude that alligators perform better in a spatial learning task at 22ϒC, than at 32ϒC. The results of this study present new data and a novel approach to quantifying learning in a predatory reptile.

Ecological factors such as food type and the size of social groups can influence the cognitive abilities of ectotherms in the same manner as they influence endotherms such as mammals and birds. On the other hand, ecological factors such as temperature and climate may have a very different effect on the cognitive abilities in ectotherms. Therefore, studies on ectotherms may provide new insights into the evolution of cognitive abilities because these ecological and environmental aspects will affect ectotherms in ways that birds and mammals are insensitive. American alligators make a useful organism for cognitive studies because they share ecological factors with birds and mammals (such as parental care and predatory behaviors). However, as ectotherms they offer unique

insights into the evolution of complex behavior, adaptability to novel situations and the effect of environmental pressures on the evolution of cognitive abilities. To push these results to an extreme conclusion, one could relate our results to current trends in global climate change and the impact of temperature changes in the alligator habitat that are too sudden to permit adaptation. As a top predator in their ecosystem, any change in numbers or physiological performance would upset the balance of other species.

Chapters 4 and 5 investigated the unknown effect of organochlorines on the spatial learning ability, as well as some of the aspects of metabolism and the thermoregulatory system of hatchling American alligators. Residual levels of DDE contaminate the waters inhabited by American alligators, and the effect of these pollutants on development, survival and species preservation needs to be explored. We determined whether learning performance and standard metabolic rate or preferred body temperatures are perturbed by DDE exposure because such changes could affect the overall health of animals, their reproductive success, and the health and growth rates of hatchlings. Additionally, given that altered thyroxin levels have been found in juveniles exposed to DDE, it is also critical that research efforts address any metabolic disturbances or effects of DDE on metabolic parameters in American alligators.

Chapter 4 describes the results of experiments looking at the effects of DDE on cognition of American alligators by comparing the performance of individuals that were exposed as embryos to an organochlorine to the performance of control individuals in a spatial learning task. Alligators exposed to

DDE through their origin at the polluted Lake Apopka, committed a greater number of errors while performing in a learning task. However, this group also showed a significant improvement in the number of errors committed during a second reversal. DDE exposure appears to affect the acquisition of a task, however it does not seem to affect the utilization of this task. This implies that such exposure alters the ability of these animals to learn and acquire an association between a physical task and a stimulus. However, this exposure does not seem to perturb an animal's ability to utilize this association in a novel situation. In other words, DDE is detrimental to an animal's ability to create new memories but does not seem to disrupt the ability of animals to use these memories. This is an important finding because such an effect could upset a variety of ecologically important behaviors.

In Chapter 5, we measured a variety of metabolic parameters including, heat production, SMR and CO_2 production. Results from these metabolic measures imply that no difference was seen between individuals exposed to DDE and individuals that had not been exposed to DDE. Finally, Chapter 5 also presented data that shows that DDE exposure alters the preferred body temperature of hatchling alligators. If this altered preference persists into adulthood it could affect the health of the individuals and their offspring, indirectly leading to changes in population dynamics. Numerous aspects of piokilotherm metabolism are affected by environmental temperature. If the altered preferred body temperature we observed in hatchlings exposed to DDE persists, it could indirectly alter several of these temperature sensitive aspects of the animal's

physiology. Such altered physiological parameters could therefore affect the health and persistence of this species.

Overall, we conclude that training and performance of visual and spatial discrimination abilities develop early in alligators, as young as 8 weeks posthatching. Furthermore, the visual and spatial learning in an individual do not require imitating a parent, but could be innate or epigenetic traits. Also ecological factors such as temperature affect cognitive performance. Specifically, our animal's performance in a learning task was improved at a lower temperature. However, the current investigation was not designed to determine if temperature has a differential effect on memory acquisition or memory utilization. Future studies could tease apart these two elements of this specie's discrimination abilities. *In ovo* organochlorine exposure on the other hand affected the memory acquisition element of the American alligators discrimination ability. Finally, while organochloride contaminants do not appear to affect metabolism of the hatchling following exposure *in ovo*, such contaminants appear to affect preferred body temperature. Such changes may indirectly upset the ecosystems these top predators inhabit.